Arbuscular mycorrhizal fungi and their influence on growth and water relations of sweet cherry rootstock and tomato plants

By

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Quote

وَلَسَوْفَ يُعْطِيكَ رَبُّكَ فَتَرْضَىٰ (٥)

And indeed your Lord will soon give you so much that you will be pleased. *[Surah Al Duha 93:5]*

Declaration

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Abstract

This thesis presents a literature review and results of experimental studies related to the role of arbuscular mycorrhizal fungi (AMF) in plant water relations in sweet cherry and tomato (as a fast-grown model system). As AMF can assist host plants to increase water relations during drought stress, they may also help plants to moderate negative impacts of excess water, such as the splitting of soft fruit. The experimental chapters focus on two main areas. Firstly, the diversity and abundance of AMF in a conventional and organic sweet cherry orchard were investigated with a preliminary study. Secondly, the impact of AMF on cutting survival in sweet cherry, and growth and physiological functioning in both sweet cherry and tomato were assessed in several experiments. In all studies the level of phosphorous supplied to plants was low to encourage AMF colonisation and the amount of root colonisation was determined to ensure a substantial difference from the controls.

To determine abundance and diversity of AMF in an organic and conventionally managed cherry orchard in two seasons, spores were extracted from soil samples, counted and spore morphology was examined by microscopy. Spore characteristics included three categorical variables (shape, colour, and pattern) and two continuous variables (spore diameter and the number of spore wall layers). The average number of spores in the conventional orchard soil was significantly higher than the organic site in both seasons. Canonical discriminant analysis of spore characteristics revealed that there was substantial overlap in the characteristics between each site and season, most likely reflecting a high similarity of species diversity.

An objective of this study was to determine if the presence of AMF '*Rhizophagous irregularis*' and local AMF species on sweet cherry rootstocks results in greater survival and establish more rapidly from cuttings inoculated with AMF than those not inoculated, and to have better regulation of water balance under conditions of water stress than for plants without mycorrhizae. It was found that inoculation with *Rhizophagus irregularis* (syn. *Glomus intraradices*) significantly increased the survival rates of cherry cuttings; however the effects on growth (height, stem diameter, leaf number and biomass) and on physiological

variables (photosynthesis rate, stomatal conductance and leaf water potential) were limited. Treatments of low water exposure (50% of water holding capacity) followed by high water (150% of water holding capacity) were applied to established cherry plants. Local soil inoculum was used in an additional experiment and field soil was filtered to remove spores so that a complementary control treatment could be applied. Surprisingly, plants grown in the filtered soil had significantly higher AMF colonisation than unfiltered soil, and unfiltered soil had higher bulk density than filtered soil. The results showed that sweet cherry grown in filtered soil had significantly higher growth, photosynthetic rate and stomatal conductance than those grown in unfiltered. Due to the confounded effects of colonisation and bulk density, the role of AMF in these physiological responses is difficult to elucidate.

Similar studies with tomato plants aimed to determine if the presence of mycorrhiza tomato plants would lead to better regulation of water balance under conditions of water stress than for plants without mycorrhizae. Tomato seedlings inoculated with *R. irregularis* showed enhanced growth in terms of height, number of leaves and biomass, compared with controls. Further, AMF tomato plants generally showed higher photosynthetic rate, stomatal conductance and an increased rate of transpiration which indicates better regulation of water balance under conditions of water stress than for plants without AMF. Therefore, the response of plants to AMF was more apparent for tomato than sweet cherry.

In conclusion, several hypotheses were tested with experiments in this thesis to explore the role of AMF in sweet cherry and tomato growth and physiological functioning, particularly in relation to water fluctuations. While there were some experimental limitations, results reflected that AMF can assist plant establishment and potentially provide benefits to soft fruit crops to assist mediation of water stress.

Communications arising from this thesis

Journal publication

Mohamed, H, Barry, K, Measham, P. The role of arbuscular mycorrhiza in establishment and water balance of tomato seedlings and sweet cherry cuttings in low phosphorous soil. Acta Horticulturae, under review.

Conference publication

Mohamed, H, Barry, K, Measham, P 2014. The role of arbuscular mycorrhiza in establishment and water balance of tomato seedlings and sweet cherry cuttings in low phosphorous soil. Presented poster, in Proceedings of the International Congress of Horticulture, Brisbane, Australia, 17-22 August 2014.

Communication to industry

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Seminar

Hend Mohamed 2013. The impact of mycorrhizal fungi for balancing water uptake of tomato and sweet cherry (*Prunus avium L*). School of Land and Food, University of Tasmania, 5th June 2013.

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Chapter 1 Introduction

Arbuscular mycorrhiza fungi (AMF) are beneficial fungi that associate with plant roots and are the most widespread underground obligate mutualistic fungi. Over 80% of terrestrial plants can form symbiotic relationship with AMF in the natural environment (Dodd 1992; Eom et al. 2000; Parniske 2008). The relationship between AMF and the roots of plants has long been associated with improved plant performance (Kaya et al. 2003). Colonisation of plant roots with mycorrhizal fungi greatly increases the "effective" root surface area for water and nutrient uptake by plants, and previous research in other crops has shown that colonized plants are more drought tolerant (Augé 2001). However, little is known about symbiosis in perennial systems such as sweet cherry, or about the impact of AMF on the ability of sweet cherry trees and fruit to tolerate excess water.

Sweet cherry is a high value perennial crop grown in most states of Australia. Tasmania has the benefit of a climate well suited for production of quality cherries that mature over the mid-December to late-February summer period, but are also likely to experience late summer rainfall when fruit is at the most susceptible stage for rain-induced fruit cracking (Measham et al. 2014; Puniran et al. 2010). Sweet cherry fruit is sensitive to changes in water uptake; cherry trees, when exposed to excess water late in the growing season exhibit severe yield losses from fruit cracking. Rapid and excess water uptake via the root system induces fruit cracking (Measham 2011; Measham et al. 2012; Measham et al. 2014). In Tasmania, yield losses due to cracking have been recorded as up to 54% (James et al. 2011; Measham 2011). This is a major concern for growers.

As AMF can alter the water uptake patterns in host plants (Asrar et al. 2012; Augé 2001), there is the possibility that AMF may influence water uptake, and therefore the incidence of cracking, in cherry trees. Most AMF-inoculated plants have shown higher stomatal conductance and an increased rate of transpiration, increased size, and increased tolerance of water stress by increasing root hydraulic conductivity (Augé 2001). AMF symbiosis can spread extraradical mycelia outside the roots to have access to a greater quantity of water and soil minerals for increased water uptake and nutrient absorption for the host plants. In return,

the symbiont receives plant carbohydrates for the completion of its life cycle (Bonfante et al. 2010a; Parniske 2008). According to a number of studies, AMF can control water in both extremes of water availability: drought and abundant water conditions (Augé 2001; Birhane et al. 2012; Koltai et al. 2010b) as can be experienced in orchards when summer rainfall occurs after a period of warm, dry weather.

It has been shown that AMF-inoculated strawberry and tomato plants, when subjected to drought conditions, have higher transpiration and stomatal conductance, as opposed to non-inoculated plants (Borkowska 2002; Subramanian et al. 2006). Fini et al. (2011) revealed that AMF can affect the morphology of the roots of inoculated plants by increasing root growth and amplifying the amount of water accessible in the soil. Furthermore, since mycorrhizal symbiosis can establish naturally in the soil and assist plants to respond to low water conditions by scavenging water more effectively, the AMF symbiosis could maintain water balance and assist the plant to open their stomates to improve carbon gain under drought conditions (Augé 2004; Fini et al. 2011). Maintenance of uniform water status is important for soft fruit such as cherry. It has been found that regulated and uniform water can increase skin elasticity (James et al. 2011) and therefore the ability to withstand rapid and excess water uptake. Herbaceous plants such as tomato are also sensitive to changes in water availability (Wahb-Allah et al. 2011). The tomato is also a soft fruit which suffers from water-related disorders such as fruit cracking caused when there is rapid water uptake into the fruit (Peet 2008), therefore this study into the impact of AMF on water uptake has potential benefits for all soft fruits

The studies in this thesis were designed primarily to explore the role of arbuscular mycorrhizal fungi (AMF) in horticulture systems; in particular their effect on water uptake. This study was undertaken in Tasmania and focused on sweet cherry trees, while tomatoes were also used as a model system. The impact of AMF colonisation on plant establishment, growth and function was also assessed in sweet cherry and tomato. In Tasmania, there is currently no information available about which AMF are dominant in agricultural or horticultural landscapes, including sweet cherry orchards. Although it is expected that *R. irregularis* is present in Tasmanian soils, given its ubiquitous distribution globally, it may not

be the most common AMF species associating with sweet cherry plants in southern Tasmania. For this reason, it is relevant to use locally abundant species in studies which have implications for practical management. In one study presented in this thesis, local orchard soil was used. Also, as a preliminary investigation, the AMF spore types in the rhizosphere soil of a conventional and organic orchard were characterised and counted, across two seasons.

Therefore, this thesis primarily aims to investigate the interaction between AMF and sweet cherry and between AMF and tomato and its effect on plant growth and water relations. In the absence of established orchard trees bearing fruit with and without AMF colonisation, experiments were carried out with plants of pre-fruiting age in glasshouse trials using either commercial AMF inoculum or local field soil as inoculum. An exploration of local AMF abundance and diversity was also conducted with microscopy of spores. Detailed aims and hypotheses are presented in the introduction of the three experimental chapters.

Chapter 2 Literature review

2.1. Background context and purpose of the literature review

Arbuscular mycorrhizae fungi (AMF) are from the order Glomales, of the phylum Glomeromycota, and are the most widespread obligate fungi known to form mutualistic relationships with plant roots. The Glomeromycota phylum has ten genera, and *Glomus* is the largest genus in the phylum (Redecker et al. 2006). AMF is an ancient symbiosis that originated at least 460 million years ago, based on the fact that colonised fossil roots have been observed in Aglaophyton major and Rhynia, which are ancient plants possessing characteristics of vascular plants (Fulekar 2010; Smith et al. 1997). AM symbioses were present in the early ancestors of extant land plants structures resembling vesicles and spores of present *Glomus* species (Fulekar 2010). It was first demonstrated that mycorrhizal plants grew faster than non-mycorrhizal plants in the 1940's in Japan (Koide et al. 2014). After that, in Europe, in 1957, a study of the function of the symbiosis was made by Mosse showing that arbuscular mycorrhizal sporocarps of *Endogone (Glomus) mosseae* infection led to improved growth of apple seedlings and clonal leaf bud cuttings in autoclaved soil (Koide et al. 2004). AMF have to form symbiotic associations with host plants to complete their life cycle (Smith et al. 1997). The term "mycorrhiza" is derived from the Greek, myco- means fungi and -rhiza means root (Bucher et al. 2009). Jones et al. (2004) stated that the term mycorrhiza was coined to show the symbiotic relationship between fungi and the roots of plants.

The name "arbuscular" is derived from the characteristic structure of branched hyphae within the cortical cells, called arbuscules (Smith and Read, 1997). Vesicles may also be formed between or within the cortical cells (Smith et al. 1997). AM fungi were first discovered and described in the late nineteenth century (Brundrett 2004; Smith et al. 1997). AM fungi receive carbohydrates from host plants while plants benefit from mycorrhizal fungi through improved nutrient uptake (including elements otherwise immobile and unavailable to plants), tolerance to diseases, enhanced water relations and decreased uptake of heavy metals. Such benefits have been demonstrated by a multitude of inoculation studies e.g. (Augé 2001; Birhane et al. 2012; Bolandnazar et al. 2007; Kaya et al. 2003; Rutto et al. 2002)

This literature review will explore the relevance of mycorrhizal fungi in horticulture systems, especially in the potential alleviation of stress in sweet cherry and tomato. The review will begin by outlining what mycorrhizal fungi are and their benefits to plants in general. Then the review will focus particularly on cherry and the key stresses experienced in commercial cherry orchards. However note that there is little literature yet on mycorrhizal fungi of sweet cherry, which is a clear research gap.

2.2. The types of arbuscular mycorrhizae fungi

There are two main types of mycorrhizal fungi, including the endomycorrhiza (which include arbuscular mycorrhizal fungi, or AMF), and the ectomycorrhiza (Smith et al. 1997) and the morphology of each is outlined in Figure 2.1. AMF belong to the phylum Glomeromycota, which has four orders *Glomerales*, *Diversisporales*, *Archaeosporales*, and *Paraglomerales.* A recent classification placed 25 genera in the Glomeromycota (Bainard et al. 2011). Over 250 species are known to exist, based on spore morphology belonging to genera such as *Gigaspora*, *Scutellospora*, *Glomus*, *Acaulospora)* (Bainard et al. 2011; Brundrett et al. 2013), while only about 150 AMF species have been described thoroughly (Oehl et al. 2003).

Characteristics of endomycorrhizal fungi include fungal material that is external to roots (extra-radical hyphae) and also that which penetrates the cortical roots (intra-radical hyphae) forming abuscular hyphae. Hyphae are the filamentous vegetative structures of the AMF, and a network of hyphae is known as mycelium. The taxonomy of AMF depends on two main categories of arbuscles, which are the "Arum" and "Paris" type. The differences between them depend on how arbuscles form inside the cortical cell. In the "Paris" type hyphae grow as coils while in "Arum" they are linear (Brundrett 2004).

Ectomycorrhizal fungi have a thick mantle that surrounds the roots of host plants by an outer sheath of hyphae which may increase the absorbing surface area of the host plant (Mukerji et al. 2000; Sharma et al. 2010). The penetration of ectomycorrhizal fungi is restricted between outer cell layers without going within the cell membrane of the cortical cells, and hyphae push their way mechanically by forming specialized nutrient transport tubes (Mukerji et al. 2000; Sharma et al. 2010). Ectomycorrhiza can be distinguished by a "hartig net" with

labyrinthine hyphae between root cells (Brundrett 2004). The most common species are from the phyla Basidiomycota and Ascomycota while some species are placed in the Zygomycota (Brundrett 2002; Sharma et al. 2010).

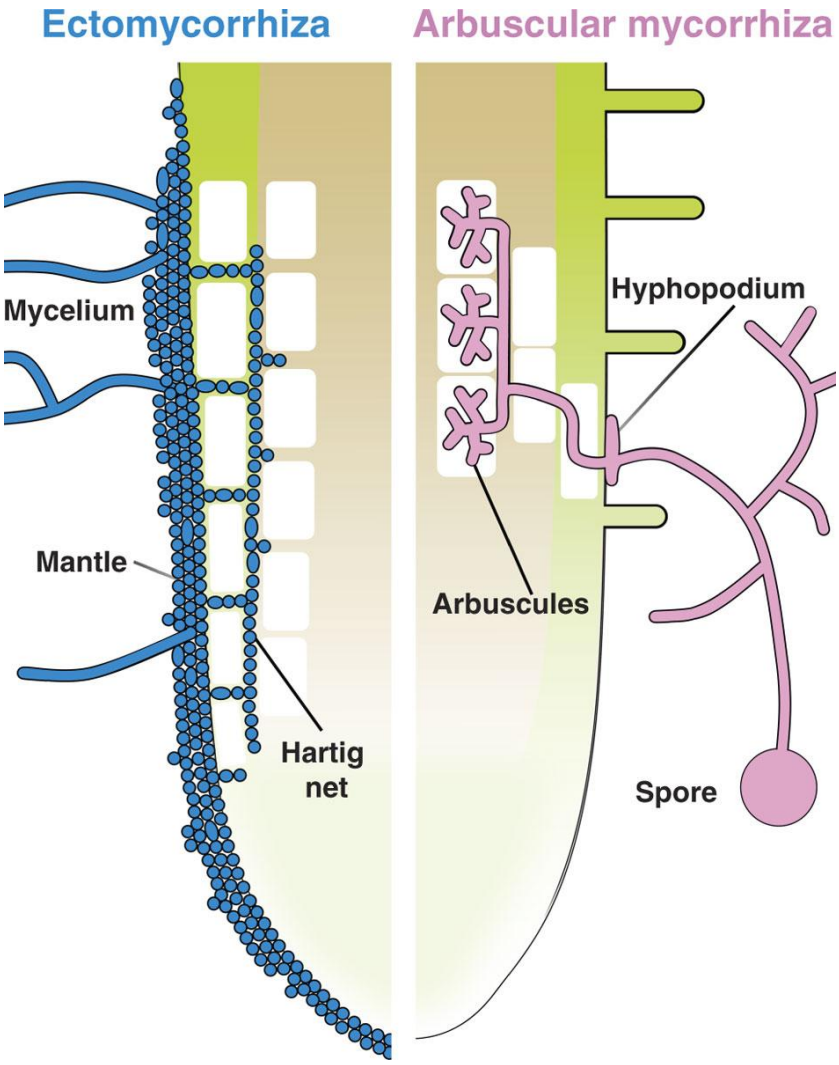

Figure 2.1: The difference between ectomycorrhizal and AMF fungi in terms of morphological features in the interaction with a plant root tip (Bonfante et al. 2010a)

2.3. The life cycle of AMF

During their life cycle, AMF, unlike many groups of fungi, do not reproduce sexually. Their life cycle begins with germination of an asexual spore in the soil (Bago et al. 2000; Parniske

2008) as outlined in Figure 2.2. Firstly, hyphal growth from spores form a hyphopodium (like an appressorium) on the surface of the host plant root. Then, these hyphae penetrate into the epidermal and cortical cells, where the absucular structures form inside. After that, hyphae grow out into the soil forming a branched hyphae that plays a key role in exploring the soil and transporting nutrients and water to the host plant. Finally, new asexual spores are formed by the internal, and in some species also by external hypha, which continues the life cycle (Bago et al. 2000; Parniske 2008).

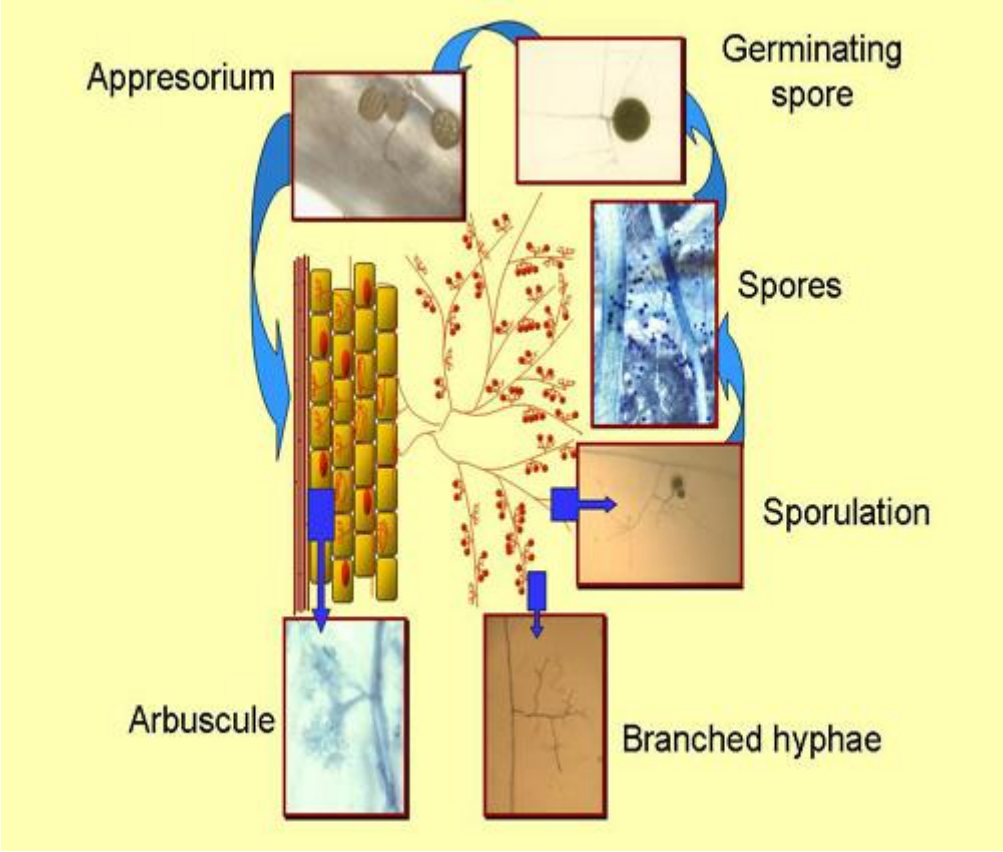

Figure 2.2: Life cycle of arbuscular mycorrhizal fungi (Bago et al. 2000)

2.4. Establishment of AMF

The association between arbuscular mycorrhizal fungi and host plants occurs for many plant species (more than 80% of terrestrial plants) and includes fruit trees. The association may

become established naturally in the nursery or when transplanted (Calvet et al. 2004; Eom et al. 2000; Fulton 2011). Most AMF species are known to be non-specific for host plant species. Therefore, a mixture of different AMF species may associate with a plant species and even an individual plant at any one time in nature (Dodd 1992; Eom et al. 2000; Parniske 2008).

Colonisation by different mycorrhizal species leads to enhanced nutrient and water uptake as well as improved disease resistance in host plants (Eom et al. 2000; Fulton 2011; Hartnett et al. 1999). The relationship between fungi and host plant differs depending on plant species, and these differences could reflect the structure and pattern of colonisation (Eom et al. 2000; Fulton 2011). Additionally, the symbiotic relationship between mycorrhizal fungi and the host plant decreases significantly with increasing levels of available phosphate. In addition, higher plant water and P uptake have been detected when more extraradical hyphae is present on roots, compared with non-mycorrhizal plants (Garcia-Garrido et al. 2009; Parniske 2008).

In addition to one plant being colonized by several AMF species at once, the same individual mycorrhizal fungus can colonize several plants at the same time, forming what is known as a common mycorrhizal network able to transfer carbon, nitrogen and phosphorus between plants (Diédhiou et al. 2010; Fulton 2011).

The next section of this review will include what is known about the structure of the relationship between AMF and host plants with respect to pre-colonisation, external mycelial formation and internal factors relating to structure of the AM fungus during the root colonisation. This is relevant given studies presented in this thesis will involve inoculation of plants with AMF and several factors may influence how AMF develop.

2.5. Mechanism of AM fungal root penetration and factors influencing colonisation

Plant roots can be inoculated with mycorrhizal fungi in three ways: via spores, infected root fragments or hyphae. Spore inoculum is typically the best way to identify the species accurately prior to colonisation (Smith et al. 1997). Spores begin the germination process by producing short explorative mycelium. This is stimulated by a hormone called branch factor

(strigolactone) that the host plant exudes as a signal when under stress (Bonfante et al. 2010a; Parniske 2008). It has recently been demonstrated that high levels of available P in the soil results in less exudation of strigolactone, and in turn less germination of AMF spores (Balzergue et al. 2011). After the explorative mycelium meets a root hair, a hyphopodoium forms on the root surface and hyphae then invades the epidermal cells, as seen in Figure 2.3. The fungi produce mycorrhizal factors as signals ("Myc" factors) that are induced by calcium spiking in root epidermal cells. These signals lead to activation of cellular and transcriptional responses in the host plant which allow colonisation to continue. Hyphae then penetrates the cortical cells of the root and forms the arbuscules (Douds Jr et al. 1999; Parniske 2008).

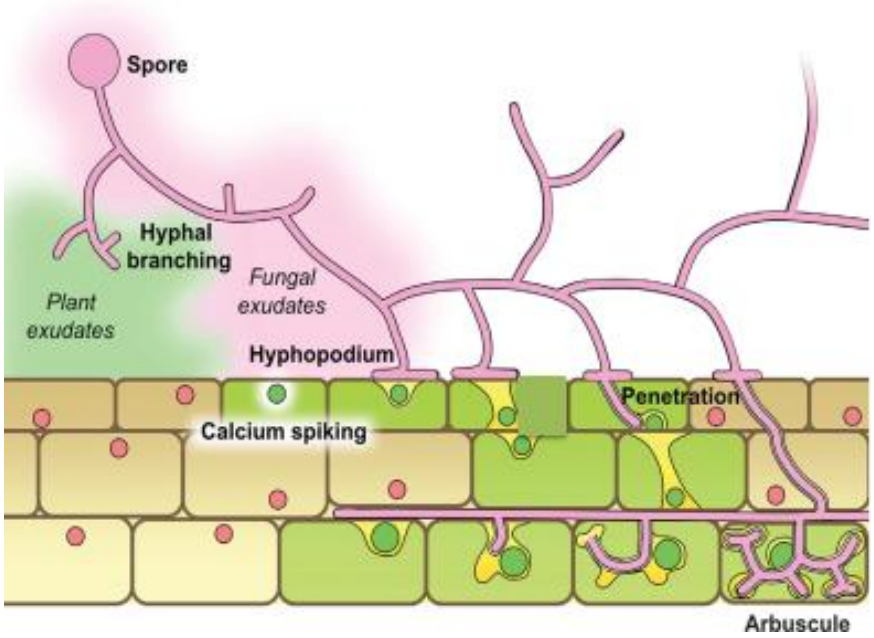

Figure 2.3: Mechanism of AM fungal root penetration modified from (Bonfante et al. 2010b)

The arbuscles are the interface between the mycorrhiza and roots and are responsible for the exchange of carbon that the fungi require for energy. In exchange the host plants receive several benefits, including improved water relations, enhanced nutrient uptake that is superior to non-mycorrhiza plants, increased pest and disease resistance and modified root morphology (Douds Jr et al. 1999). Ruiz-Lozano (2003) states that AMF inoculated plants reduce the symptoms of stress in plants, and improve their ability to uptake nutrients and resist drought. The morphology of the root system is important for nutrient uptake, water

absorption and growth. Roots inoculated with spores are not always successfully colonized because the colonisation depends on the root's strength. For example, short, weak and poorly developed roots frequently respond well, while long fine roots do not respond to mycorrhizal colonisation as well (Smith et al. 1997). Furthermore, the ability of AM to colonize roots under drought stress is lower than when plants have ready access to water (Ruiz-Lozano et al. 1995). These are factors that may influence colonisation success.

2.6. The impact of AMF on nutrition of host plants

Arbuscular mycorrhiza are widespread in the soils throughout the world (Linderman 1988). Marschner et al. (1994) suggest that mycorrhiza enhance growth through nutrient uptake by external hyphae that can reach more than 10 cm from the root. External mycelium is important for exploration of soil pores and interaction with organic matter in the soil. It is also important in establishing soil aggregates, which are important for healthy soil structure (Ridsdale 2012; Smith et al. 1997). This means that both the host plants and soil gain benefit from establishing relationships with fungi (Ridsdale 2012; Smith et al. 1997). AM fungi can supply around 80% of plant P , 25% of plant N , 10% of plant K, 25% of plant Zn and 60% of plant Cu (Marschner et al. 1994). Moreover, plants depend heavily on mycorrhizal fungi in low P soil for improved P and N uptake . In contrast, high amounts of available P leads to suppression of the relationship between the fungus and host plant as well as N_2 supply. AMF supply host plants with about 25% of the total plant N_2 compared with 3.5% of N_2 supplied via non-inoculated plants (Fulton 2011; Marschner et al. 1994; Porcel et al. 2004).

Increased uptake of nutrients by AM fungi is attributed to the ability of the fungi to transfer nutrients from soil to the plants beyond the "depleting zone" around the roots, and also to their ability to degrade complex organic and inorganic material by secreting extracellular enzymes or organic acids (Estrada-Luna et al. 2000). Phosphate is generally immobile in soil (Ridsdale 2012) and since there is a greater reduction in the mobility of P and other nutrients in dry soil, AM symbiotic relationships assist plant acquisition (Smith et al. 1988). As a result, plants colonized with mycorrhiza typically have higher P concentration per dry weight in the shoots and enhanced root length (Douds Jr et al. 1999). According to Douds Jr et al. (1999), the AM fungi exude phosphatase enzymes into the soil for hydrolysis and facilitate

absorption by extraradical hyphae, and transfer P to the host plant. In contrast, it is mentioned that increased P concentrations will typically lead to reduced hyphal growth (Helber et al. 2011).

Moreover, Calvet et al. (2004) reported that plants inoculated with *Glomus intraradices,* now known as *Rhizophagous irregularis* (Schüβler et al. 2010), acquire more nitrogen into their shoots. Leigh et al. (2009) stated that nitrogen in the soil has two forms (from organic and inorganic sources), so the extraradical hyphae of *Glomus* sp. can metabolize both forms of nitrogen by glutamate syntheses activity. Plants can capture N in inorganic forms such as nitrate or ammonium which is highly mobile in the soil matrix. In contrast, most N occurring naturally in the ecosystem is structured in complex organic compounds and dispersed randomly in the soil. These complexes make N unavailable to the plants. Consequently, AM fungi play a core role in supplying host plants with N, since their hyphae extensively colonize the soil, in order to isolate N from organic material and transfer it to the host plants (Leigh et al. 2009).

Plants deliver glucose as the source of carbon to the AM fungi, which subsequently consume about 4-16% of the photosynthetic carbon production to maintain their activity; and increased carbon demand may lead to higher rates of photosynthesis (Kaschuk et al. 2009). Jones et al. (2004) stated that increased photosynthesis could result from increased sink strength induced by the AM fungi. Kaschuk et al. (2009) advocated that AM fungi enhance plant photosynthesis through N and P acquisition, thereby improving plant growth and yield. According to Birhane et al. (2012), *Boswellia papyrifera* seedlings have a higher biomass than control seedlings, increased leaf size and imperoved uptake of P leading to enhanced photosynthesis and stomatal conductance. Early inoculated guava plantlets showed enhanced growth in terms of larger shoot length, leaf area and root and shoot biomass (Estrada-Luna et al. 2000).

2.7. The impact of AMF on plant water status

Water status plays an important role for plants in terms of growth and physiological development (Augé 2001; Fulton 2011; Khalvati et al. 2005). Enhanced water intake of AMF

plants during drought stress, compared to non-AMF plants, could occur because hyphae can penetrate the small pores which are inaccessible to most plants roots, thus enabling greater soil water extraction. This is because root hairs attain a diameter of up to 10- 20 µm, while fungal hyphae, which are 2-5 µm in diameter, can access finer pores in the soil (Al-Karaki 1998). Efficient use of water by mycorrhizal plants under water stress results in the stomata in the leaves remaining open for longer relative to un-colonized plants (Augé 2001), and this results in improved production (Al-Karaki 1998; Augé 2001; Fulton 2011). Furthermore, it was found that relative water content in AMF-inoculated maize leaves was higher than that of non-mycorrhizal plants, under conditions of differing salinity levels (Subramanian et al. 1995). Moreover, the colonisation of mycorrizal fungi improves the efficiency of water consumption (Augé 2001; Fulton 2011; Sheng et al. 2008). AM fungi can alter the water status of the host plants because the amount of the water that extraradical hyphae can obtain is up to 100 nl H_2O h^{-1} per hyphal infection point (Khalvati et al. 2005; Ruiz-Lozano et al. 1995).

2.7.1. Role of AMF in host plants during drought conditions

Drought stress is believed to be one of the most important abiotic factors preventing plant growth and yield in many areas (Porcel et al. 2004). Abscisic acid (ABA) is a stress hormone which moves through the xylem from the root to the different parts of the shoots where it regulates transpirational water loss and leaf growth (Hartung et al. 2002) and ABA is typically increased in drought affected plants. Drought stress has a significant negative impact on plants such as inhibition of photosynthesis associated with stomatal behavior which is partly controlled by ABA (Apel et al. 2004; Augé 2001; Quilambo 2004a; Quilambo 2004b). During drought the reduced leaf water potential leads to stomatal closure (Augé 2001). AMF symbiosis could alter the rate of water movement into, through and out of host plants. Consequently, AMF can affect tissue hydration and physiology in host plants (Ruiz-Lozano 2003; Smith et al. 1997). Drought stress resistance has been found in roots and shoots of soybean plants inoculated plants with AM symbionts (Bolandnazar et al. 2007; Fulton 2011; Porcel et al. 2004; Wu et al. 2006). In addition, drought-affected mycorrhizal soybean plants had higher leaf water potential and biomass production than non-mycorrhizal plants (Porcel et al. 2004). Al-Karaki (1998) reports that mycorrhizal wheat plants used less water to

produce one unit of shoot under drought stress. The benefits to inoculated plants are more evident under drought stress than when water is adequate. Basically, the higher photosynthetic rate in mycorrhizal plants leads to increased carbohydrate production. It has been shown that mycorrhizal barley plants, under drought stress, have carbohydrate concentrations that are about 80% greater than non-mycorrhizal plants (Khalvati et al. 2005; Porcel et al. 2004; Wu et al. 2006).

As mentioned earlier, the association between AMF and roots of host plants under drought conditions improves productivity partly by enhancing nutrient uptake, especially phosphate (Fulton 2011). Additionally, mycorrhizal wheat plants showed enhanced P uptake that may result from using water more efficiently (Al-Karaki 1998). Water-use efficiency of productivity (also called integrated water use efficiency) has been defined as "the ratio of biomass produced to the rate of transpiration" (Tardieu 2013). It was found that mycorrhizal plants that were irregularly watered showed greater water use efficiency than non-inoculated plants (Augé 2001; Fulton 2011). The symbiotic relationship improves water uptake by allowing mycorrhizal plants in dry soil to sustain higher stomatal conductance and leaf turgor by effectively providing roots access to more of the soil water reservoir (Augé 2004). As a result, AM fungi can protect host plants against drought conditions, and increase the growth of roots and shoots under shortage of water and phosphorus (Quilambo 2004c).

It has been shown that mycorrhizal plants can produce more roots than non-mycorrhizal plants under drought stress, which may lead to increased water transport (Al-Karaki 1998). This explains why higher water use efficiency occurs in inoculated plants. Ruiz-Lozano et al. (1995) found that the benefits of the extraradical mycorrhizal lattice might be greater during drought than at other times, due to an increase in the water absorption area by extraradical hyphae, and enhanced leaf gas exchange rate, water use efficiency, transpiration and increased leaf water content than non-mycorrhiza plants. Bolandnazar et al. (2007) reported that leaf area and biomass of onion plants increased after they were transplanted to soil inoculated with AMF. In these plants, stomatal conductance and leaf growth rate in mycorrhizal plants under both adequate water and water deficit are higher than non-mycorrhizal plants (Bolandnazar et al. 2007).

Several recognized processes (e.g. ABA and proline accumulation) have been proposed to assist AMF inoculated plants to better withstand drought. Mycorrhizal plants show increased production of a non-protein amino acid called proline, which forms in the majority of plant tissues experiencing drought (Hartung et al. 2002; Ruiz-Lozano 2003). Although the accumulation of proline in plant tissues has been observed in mycorrhizal plants subjected to drought, it has also been shown that mycorrhizal colonisation and drought interact in modifying free amino acid and sugar pools in roots (Ruiz-Lozano 2003). ABA has roles in altering stomatal conductance that can assist plant performance during water stress. This was confirmed in a seeding study of *Vigna ungiculata* and *Capsicum annuum* where ABA was lower in AMF plants under drought conditions (Duan et al. 1996; Estrada-Luna et al. 2000).. Therefore, mycorrhiza actually enhance leaf conductivity and afford osmotic adjustment by keeping stomata open longer to fix carbon more efficiently (Quilambo 2004c; Ruiz-Lozano 2003; Smith et al. 1997; Wu et al. 2006). Accordingly, mycorrhizal plants enhance the water uptake ability of the host plants by increasing leaf and turgor potentials, and increasing root growth (Augé 2001; Subramanian et al. 1995; Wu et al. 2006). Subramanian et al. (1995) report that AM maize plants maintained higher values of leaf water potential during a three week period of sustained drought conditions.

Mycorrhizae may have the ability to enlarge roots and their "effective" surface area so as to better utilize available water resources (Subramanian et al. 1995). However, some studies suggest that rapid extraction of water, combined with increased rate of transpiration, may lead to a sharp decline in the amount of water available in the soil more quickly (Subramanian et al. 1995). Under drought conditions, stomata start to close (stomatal resistance) in order to prevent the leaf-water status from dropping to critical levels (Bago et al. 2000; Birhane et al. 2012; Subramanian et al. 1995; Wu et al. 2013). This results in an increase in the rate of photosynthesis, so as to maintain high levels of carbohydrate, which is needed to help the plant survive during the drought conditions and recover faster after mitigation from the stress (Birhane et al. 2012; Fulton 2011; Subramanian et al. 1995). Studies show that green leaf area normally decreases under drought condition; however, AM plants more often maintain higher green leaf area than non-mycorrhizal plants (Fulton 2011;

Subramanian et al. 1995). Subramanian et al. (1995) point out that the comparative increase in green leaf area in AMF plants under drought stress occurs as a result of nitrogen acquisition by external hyphae that lead to accentuated protein in the leaf. The benefits of AMF under drought conditions might be less pronounced because of a decline in the rate of colonisation (Al-Karaki 1998). However, many studies reported that while the symbiotic relationship is positive overall for the plant due to increase P and water uptake and the consumption of carbohydrate is compensated for by improving photosynthesis, the proportion of colonisation is not directly related to the enhancement in host plant growth (Al-Karaki 1998; Khalvati et al. 2005; Porcel et al. 2004; Rutto et al. 2002).

2.7.2. The role of AMF in host plants during excess water conditions

Every plant requires water to live, however, water-logging or water inundation in the root zone which may arise from excessive rain or irrigation in poor drainage, can cause major problems in terms of oxygen depletion of the soil by respiratory activity of soil organisms, and an increase in CO_2 and ethylene levels. This can result in tissue damage, slower growth and sometimes plant death (Atwell et al. 1999; García et al. 2008).

Rutto et al. (2002) reported that inoculating peach seedlings with AMF helped the plants to develop excess water tolerance when they were experiencing flood conditions. At the same time, they found that the concentration of P, K and Zn was significantly higher in inoculated plants. These findings seem to indicate that mycorrhizal symbiosis could assist plants, in flood conditions, to adapt better than non-inoculated seedlings due to the formation of hypertrophied lenticels that make more oxygen available to the plants (Osundina 1997).

García et al. (2008) found that long-term excess water affected growth of *Lotus tenuis* plants by decreasing root growth by 36% and increasing shoot growth by 13%, and increasing P in the soil that caused a decline in AM colonisation on the plant. After lengthy flood conditions, inoculated peach plants showed considerable difference in the shoot: root ratio comparing with non-mycorrhizal plants (Rutto et al. 2002).

Excessive water can create an environment favourable to the proliferation of diseases such as crown rot, root rot and damping off (Drenth et al. 2004). The presence of AMF can reduce plant diseases in some cases, which is further outlined in the next section.

2.8. The impact of AMF on pest and disease

Pests and disease cause serious losses of crop plants of all types. For instance, in 1996-1998, in 17 regions around the world, the potential losses (without management) to fungal and bacterial pathogens, viruses, animal pests and weeds were estimated at almost 50% of barely, soybean, wheat and more than 70% of sugar beet and cotton plants (Oerke et al. 2004). There are several ways that AMF may reduce the incidences of loss to plant disease, including through enhanced nutrition, competition for nutrients and infection sites, morphological changes to the root, chemical changes in the plant, alleviation of plant stress and change in microbial community if the plant establishes colonisation with AMF before the host plant is infected by disease (Koltai et al. 2010a).

Pests and diseases can be managed in many ways, including by cultural controls, chemical controls such as fungicides, bactericides, insecticides, rodenticides, nematicides and herbicides, host resistance or by biological controls (Albajes 1999). Typically, chemical controls are used more often than biological controls, despite the fact that the former can have adverse environmental impacts such as pollution of groundwater. Biological controls of plant diseases are safer and have reduced environmental impact compared to chemical pesticides; however, the costs may be higher and the results less effective in most cases (Brimner et al. 2003; Mukerji et al. 2000).

According to Azcón-Aguilar et al. (1997b) and Koltai et al. (2010a), pathogen infection was reduced by AMF. Since inoculated plants can recover from disease faster due to improved nutrient uptake and increased biomass, this could compensate for any root damage caused by diseases (Azcón-Aguilar et al. 1997b; Gosling et al. 2006). In addition, it has been shown that after AMF colonized host root cells, pathogens were excluded from those cells (Azcón-Aguilar et al. 1997b; Gosling et al. 2006). This could be as a result of changes in host plant hormones that, in turn, lead to changes in root biochemistry associated with plant defence

mechanisms, or changes in the rhizophore microbial community (Gosling et al. 2006; Mukerji et al. 2000). The ability of AMF to control root disease is not necessarily the same for all pathogens (Fulton 2011; Mukerji et al. 2000).

AMF can reduce the symptoms of disease caused by some fungal, bacterial and nematode pathogens (Fulton 2011; Gosling et al. 2006; Mukerji et al. 2000). This restriction of pathogen proliferation is accompanied by heightened host defence reactions. The systemic protective effects induced by AMF require the accumulation of plant defences associated with enhanced reactions in the walls of the mycorrhizal host's roots. There is evidence that cellulose is induced in the cell walls early during plant defence reactions by an accumulation of PR-1, a protein in the cell wall which plays a role in associating with plant cell wall thickness. This has been reported as occurring during resistance responses of tobacco roots to a pathogen (*Chalara elegans*) and could also restrict pathogen growth within the cortical cells of mycorrhizal roots, and increase accumulation of fluorescent compounds (Cordier et al. 1998; Mukerji et al. 2000).

There are a number of different ways in which fungi may be used as a biological control of plant diseases. Firstly, one species of fungus can be used to control another fungal disease by feeding on it or attacking it, a process called mycoparasitism. Moreover, some fungal species can produce enzymes or antibiotics that are capable of inhibiting the growth of organisms around plants. Colonisation of AMF can cover the roots of host plants thus preventing the invasion of host roots from pathogen, especially from nematodes. In addition, these fungi can increase nutrient and water uptake in the host plants resulting in improved general plant health and disease resistance (Brimner et al. 2003; Gosling et al. 2006; Hooker et al. 1994).

Table 2.1: Disease reduced by AMF (Gosling et al. 2006; Hooker et al. 1994; Mukerji et al. 2000)

Pathogen	Disease	Host
Sclerotium cepivorum	White rot	Onions (*Allium cepa*)
Phytophthora sp.	Root rot	*Citrus*
Fusarium oxysporum	Fusarium root rot	Asparagus (*Asparagus officinalis*)
		French bean (*Phaseolus vulgaris*)
Verticillium dahlia	Verticillium wilt	Tomatoes (*Lycopersicon esculentum*)
		Aubergines (*Solanum melongena*)
Helicobasidium mompa	Violet root rot	*Asparagus*
Rhizoctonia solani	Root and stem rots	Mung bean (*Vigna radiate*)
Aphanomyces euteiches	Root rot	Pea (*Pisium sativum*)
Phytophthora cinnamomi	Root rot	*Avocado*
Fusarium oxysporum	White rot	*Cuminum cyminum*
Radopholus citrophilus	Nematode	*Citrus limon*
Meloidogyne incognita	Nematode	*Avena sativa*
Pratylenchus vulnus	Nematode	*Prunus avium*
Pratylenchus vulnus	Nematode	*Prunus domestica*

2.9. The effect of AMF on tomato

The tomato is a soft fruit which suffers from water-related disorders such as fruit cracking that are caused when there is rapid water uptake into the fruit at the same time as ripening or other factors reduce the strength and elasticity of the tomato skin (Peet 2008). Subramanian et al. (2006) found that tomato plants *Lycopersicon esculentum* L. (Variety PKM-1) inoculated by *Glomus intraradices,* "today known as *Rhizophagous irregularis* (Formey et al. 2012; Sashidhar et al. 2012)", and then subjected to different levels of drought conditions in the field, had greater growth (height and number of branches) than non-inoculated plants. When the inoculated tomato plants were exposed to severe drought stress, they showed more intensive colonisation and a significant increase in the biomass of their roots and shoots under

all levels of drought conditions. This is more pronounced under drought conditions as the colonisation enhances the leaf relative water content (RWC). This colonisation also results in improved plant N and P nutritional status which in turn increased production of tomato fruit by 24% under severe drought conditions, 23.1%, under moderate conditions and 12.1% when drought conditions were mild (Asrar et al. 2012; Foo et al. 2013; Subramanian et al. 2006).

2.10. AMF diversity

Despite the recognised importance of AMF, understanding of diversity and biology in Australia, and globally, is very limited (May 2001). In the vicinity of Kakadu National Park in tropical Australia, spore surveys discovered 15 species of AM fungi and eight additional undescribed AM fungi were recovered from the same soil samples using pot-culture isolation methods (Brundrett et al. 2013). Pot-cultures were especially essential for detecting *Glomus* species that had high inoculum level, but rarely produced spores in soils (Brundrett et al. 2013). Spore surveys in Australia apparently underestimated the importance of *Glomus* species due to rarely producing spores in soils, and overestimated the activity of *Acaulospora* species which has numerous small spores. Additionally calculated spore biovolumes overestimated the importance of *Scutellospora* and *Gigaspora* species due to large spores (Brundrett et al. 2013).

2.11. Commercial production of Sweet Cherry (*Prunus avium L*)

Sweet cherry is a high value summer crop because it is favoured by consumers. As cherry consumption depends on quality, the size, flavour, firmness and the dark colour of cherry fruit is important. Productivity depends on carbohydrate levels, and regular irrigation is important to maintain quality and ideal firmness (Diédhiou et al. 2010; von Bennewitz et al. 2011). The fruit is rich in fibre, vitamin C, carotenoids and anthocyanins that help to prevent the risk of many diseases such as cancer, cardiovascular disease, diabetes, obesity (Bright et al. 2004; Diédhiou et al. 2010).

James et al. (2011) stated that sweet cherry is grown commercially in 40 countries; the main producers are USA, Iran, Turkey, Germany, and Italy. Australia has increased its cherry production in recent years, and now accounts for 0.5% of the world's total cherry productions. In 2008/09 Australia produced 9,500 tonnes, worth an estimated $95 million. Estimates of the 2014/15 season are for total production of 16,000-18,000 tonnes (P. Measham, pers. comm.). Australian exports for 2009-2010 were estimated to be approximately US $20 million. The most recent Australian figures available (Figure 2.4) show that the current top three export markets, by volume are Hong Kong, Taiwan and Thailand (James et al. 2011; Predieri et al. 2003; Webley et al. 2011).

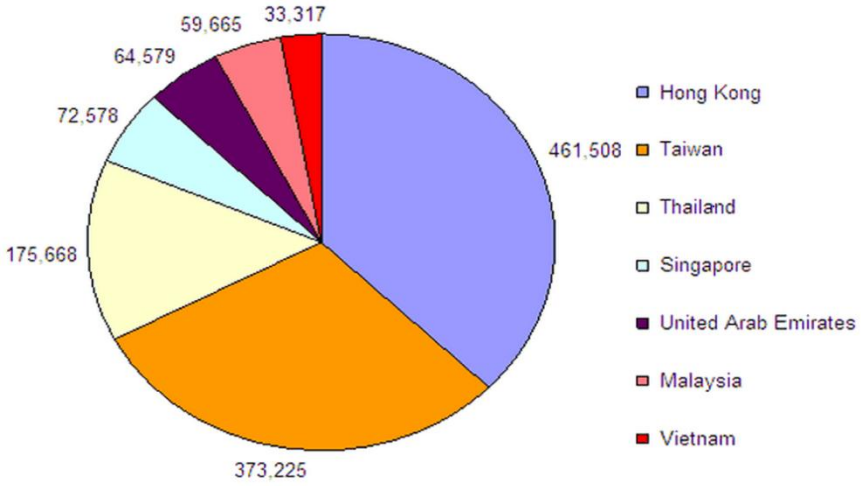

Figure 2.4: Top Australia Export Destination by Volume (kg) (Webley et al. 2011)

2.11.1. Cherry rootstocks and Key growth stages in sweet cherry

As cherries are considered to be difficult to propagate from cuttings and they do not come true to type from seed, rootstocks offer a range of benefits including modifying growth or cropping characteristics, tolerance to unfavourable soil conditions, soil borne diseases or pests and adaptation to soil or climatic conditions (James et al. 2011). There are different types of rootstocks such as Mahaleb (*Prunus mahaleb*), Mazzard (*Prunus avium*), Gisela 6 (*P. cerasus x P. canescens*), Gisela 12 (*P. cerasus x P. canescens*), Gisela 5 (*P. cerasus x P. canescens*), Krymsk 5 (*P. fruticosa x P. lannesiana*), Krymsk 6 (*P. cerasus x P. cerasus x P. maackii*) Maxma 14 (*P. mahaleb x P. avium*) and Colt (*P. avium x P. pseudocerasus*). The

last of these is considered to be the best rootstock for growing cherry trees in large gardens and community orchards, as it produces a tree with a height of 3.5m - 5m, tolerates poorer soils, and is also useful for large cherry fans, include the origin too (Long et al. 2010).

In cherry production, there are several stages before the appearance of fruit. These stages start initially with dormant buds that require cold weather during the winter to achieve a set chill requirement. Once this is reached, and in response to lengthening daylight hours, buds swell, and start to increase in carbohydrate and nitrogen levels as buds burst and activate nutrient and carbohydrate mobilisation. Bloom occurs in late winter/early spring, leading up to fruit setting (Figure 2.5) (Chapman et al. 1976; Lang 2001).

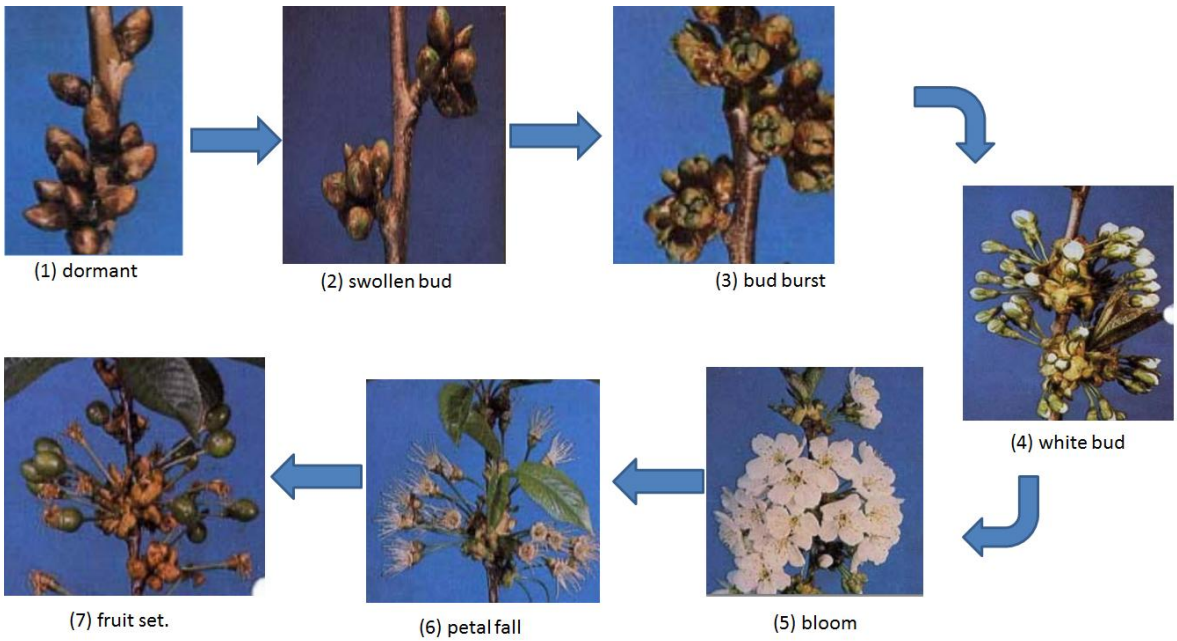

Figure 2.5: Key growth stages in sweet cherry (Chapman et al. 1976)

2.11.2. Influence of climate on sweet cherry

Sweet cherry fruits are very sensitive to climate compared to other fruit (James et al. 2011). Firstly, sweet cherry requires a number of specific climatic conditions during each key

growth stage. For example, an adequately cold winter is needed to enable the buds to fully burst. Secondly, the growing period for cherries is quite short, the period from flowering to fruit maturity being typically just two months, so here also, the climatic condition must be just right for a successful crop. Lastly, rainfall, both quantity and timing, are critical. Moreover, James et al. (2011) suggested that some rainfall in spring is needed to ensure maximum pollination and fruit set; however, low summer rainfall is essential to avoid fruit damage and minimize disease pressure. Continuous rainfall at harvest time can lead to cracking of the fruit and a reduction in fruit quality due to water uptake through the plants' vascular systems (James et al. 2011; Predieri et al. 2003).

2.11.3. Key abiotic stresses in sweet cherry

2.11.3.1. Temperature

Cherry production is influenced by a number of environmental conditions: warm weather to encourage the growth of shoots, and cold weather, between 2°C and 12°C in winter for 40-60 days, for the buds to open later in spring. This low temperature requirement limits the regions for cherry production to the temperate latitudes (Hedhly et al. 2007). Higher temperatures in early spring are required to encourage buds to shoot and flower buds to bloom. Later, these high temperatures, combined with low rainfall, must persist to protect the fruit from damage and reduce the pressure of disease (James et al. 2011; Sansavini et al. 2005).

2.11.3.2. Rainfall

Intermittent rainfall during the growing season may lead to a rapid increase in fruit size which may cause fruit cracking. This is due to a reduction in the elasticity of the skin of the fruit (James et al. 2011; Simon 2006). Studies show that if cherry plants received consistent amount of water during the seasons, the skin surface area will increase, making the skin more elastic, and so protecting the fruit from cracking (James et al. 2011; Measham 2014; Rupert et al. 1997; Simon 2006).

As far as the water requirements of the cherry trees is concerned, while water is important during every growth stage, extreme excess soil moisture for four or five months leads to a reduction in shoot and leaf growth, and a decrease in blooms as a result of bud break. On the other hand, formation of flower buds, blooms, and fruit set and development are adversely affected by low levels of soil moisture. Thus, to produce large good quality fruit requires regular watering of the trees during the growing season (James et al. 2011; Predieri et al. 2003; Simon 2006).

2.11.3.3. Wind

Wind helps plants to pollinate, and also reduces the humidity within a crop, consequently limiting above-ground diseases and pests. However, strong wind can damage trees and fruit, causing big losses in production. Furthermore, wind may lead to increased water loss from the soil that could cause drought stress in cherry plants (James et al. 2011; Predieri et al. 2003).

2.11.4. Key biotic stresses in sweet cherry

2.11.4.1 Fungal and Bacterial Disease

The most common bacterial disease in cherry trees is canker, caused by the bacteria *Pseudomonas syringae* which withers the buds and girdle the limbs of trunk often resulting in the death of the tree. Another serious bacterial disease is crown gall, caused by *Agrobacterium tumefaciens*. This leads to infection in the root or trunk, generally below ground, which may cause stunting and a general decline in the condition of the tree (Eastwell 2005; James et al. 2011; Sansavini et al. 2005).

2.11.4.2 Nematode and Insect Pests Disease

Cherry shoots and roots are vulnerable to attack by a multitude of insects (Eastwell 2005; Pinochet et al. 1995). For example, cherries are very susceptible to mites and the light brown apple moth which are common in apple orchards that damage the fruit and leaves of the trees. Pear and cherry slug infestations result in attacks the leaves. Young leaves and shoots of the cherry can also be twisted and distorted by aphids. In addition, there live in the soil numerous

species of nematodes which, when populations are low, are harmless to the plants. However, when their numbers increase, they may attack the plants. For example, the nematode *Xiphenema americanum,* can cause Cherry Rasp Leaf, resulting in a reduction in the growth and vigour of trees. This nematode may also cause the spread of viral diseases between plants, such as the Prunus stem pitting virus (Eastwell 2005; James et al. 2011).

2.12. The current state of knowledge of AMF and the sweet cherry

There have been very few studies of AMF in sweet cherry. Calvet et al. (2004) found that cherry root stock, *Prunus cerasus*, inoculated with two *Glomus* species (*G. intraradices* and *G. mosseae)* showed good levels of mycorrhizal colonisation. Calvet et al. (2004) also reported that early infection by *G. intraradices* of cherry rootstock, Santa Lucia 64, a seed selection of *Prunus mahaleb* led to increased growth and enhanced tolerance to the presence of the root-lesion nematode, *Pratylenchus vulnus,* by stimulating plant nutrition and vegetative growth in the presence of potentially damaging levels of nematodes. It has also been discovered that cherry rootstock Santa Lucia 64 is a good host to the nematode, *P. vulnus*, which reduces plant growth (Pinochet et al. 1995). However, inoculated Santa Lucia 64 cherry rootstock plants grew better than non-mycorrhizal plants. Thus the early introduction of sweet cherry roots with AMF leads to increased growth capacity as a result of increased phosphate uptake although the cherry plants were treated by AMF, and under the nematode infections (Pinochet et al. 1995). Furthermore, Reinhart et al. (2003) found that different soil biota species, including mycorrhizal fungi, can enhance the plants growth of black cherry (*Prunus serotina*). Pons et al. (1983) found that using AMF in axenically propagated plants by tissue culture methods of *Prunus avium* L. (wild cherry) could contribute to the success of this technique which have high quality healthy plants.

Although much is already known about the symbiotic relationship between mycorrhiza and host plants, there is little information on effects of mycorrhiza on response of plants to short-term excess water, and very little, in particular, concerning the sweet cherry. There is also little knowledge about which AMF species most commonly associate with sweet cherry in commercial orchards, both in Australia and other parts of the world. Greater knowledge of the role of AMF in sweet cherry may afford insight to management options which can

partially provide natural solutions to water, nutrient and pest and disease management. As there is growing interest in soil biology and reduction in use of pesticides, this is a topic of both academic and commercial interest.

Chapter 3: Diversity and abundance of arbuscular mycorrhizal fungi in an organic and conventionally managed sweet cherry (*Prunus avium* L) orchard in the Huon Valley, Tasmania

3.1. Introduction

Arbuscular mycorrhizal fungi are important mutualists known to increase plant growth in many environments. They can assist with water and nutrient uptake and play an important role in the structure, development and sequestration of carbon in the soil (Beauchamp et al. 2005; Hijri et al. 2006).

It has been found that species distribution may reflect evolutionary adaptation to different regions (Dai et al. 2013), e.g. vegetation type, soil type, climate. Diversity in different regions can be attributed to dominant AM fungal species, e.g. crop production has a homogenizing effect on AMF communities, so cropland and natural areas are similar in diversity and richness, but their relative abundance in cropland is lower (Brundrett et al. 2013; Dai et al. 2013). Roadsides are heterogeneous environments offering a wide range of niches, which favours diversity, but may also be an outcome of disturbance (Dai et al. 2013; Rosendahl et al. 2009). In terms of abundance in a Canadian study, the native grassland (prairie) revealed higher relative abundances of AMF from roadsides than from cropland (Dai et al. 2013; Oehl et al. 2004). According to Oehl et al. (2003), the highest AMF species number was found in the low-input soil, organically managed arable land with crop rotation. However, AMF species were found to be absent from intensively managed arable lands with continuous mono-cropping of maize (Oehl et al. 2003). Interestingly, Herrera-Peraza et al. (2011) found that the ability of different AMF species and strains to improve growth of coffee crops differed according to the soil type they were introduced into when artificial inoculation was conducted. Thus the strains *Glomus fasciculatum*-like and *Glomus etunicatum*-like particularly performed better in soil relatively rich in nutrients and organic matter to enhance the growth of coffee crops. In relatively poor soils, *Paraglomus occultum* and *Glomus mosseae-like* performed best, and *Acaulospora scrobiculata, Diversispora spurca, G. mosseae-like, G. mosseae* and *P. occultum* enhanced coffee growth best in Chromic soils. In addition, *G. mosseae* and *Glomus manihotis* did best in soils of medium fertility.

Over the past century, improved understanding of agricultural productivity has led to the general adoption of higher inputs of synthetic products (e.g. fertilizers and pesticides) and intensive crop production practices (Oehl et al. 2004). It has been found that inorganic fertilisers typically limit the development of AMF colonisation, whereas organic fertilisers promote them (Carpio et al. 2005). Intensive land management can also strongly influence the abundance and community composition of AM fungi leading to a low taxonomic diversity of AMF (Bainard et al. 2011; Oehl et al. 2004). This shift in AMF community composition could be due to a number of factors including disturbance of AM fungal hyphal networks, changes in soil nutrient content, altered microbial activity, or changes in weed populations. Management strategies that require lower inputs such as organic farming tend to have a greater AM fungal diversity (Bainard et al. 2011; Carpio et al. 2005; Grant et al. 2005; Ipsilantis et al. 2012; Oehl et al. 2004). Mycorrhizal fungi have generally been shown to benefit plants, but density and species of AM fungal populations, and cost benefit analyses in ecosystems vary in response to soil properties and management practices (Troeh et al. 2009). Agronomic practices that have been used in conventional agriculture such as the application of synthetic fertilizers and pesticides have shown a range of effects on AMF. The systemic fungicides benomyl and carbendazim significantly inhibited the ability of AM fungi to colonize plants (Ipsilantis et al. 2012; Schweiger et al. 1998).

According to Oehl et al. (2005), abundance of AMF spores within soil layers depends on the host plants. For example, in maize fields it was found that the number of spores decreased with increasing soil depth, and in the deeper soil layers the numerous species found frequently in the extensive grasslands were not found in the intensively managed maize fields, (e.g. *Glomus rubiforme and Glomus sinuosum).* However, the AMF community composition changed towards even deeper soil layers and surprisingly high species richness was observed even in the deepest soil layers examined (50–70 cm) *(e.g. G. etunicatum and G. aureum* and especially the *Scutellospora species* detected, *S. calospora and S. castanea).*

Abundance and diversity of AMF can also vary with season, for example in tropical rain forests AMF abundance was recorded in two different seasons (rainy and dry) with the highest number found in the dry season (Guadarrama et al. 1999; Lovelock et al. 2003). In addition, a German study found that the percentage of spores was much lower in autumn than in spring time (Oehl et al. 2003), however this was due to a problem in identification, not

actual abundance; in autumn, a major proportion of the spores were still immature and could not be identified (Oehl et al. 2005). Troeh et al. (2009) found that higher spore counts were found in poorly drained soils than in well drained soils in soybean fields.

Many perennial fruit tree systems develop extensive mycorrhizal relationships. Given the minimal soil disturbance, management practices in perennial systems are likely to have less impact on AMF diversity and abundance than annual systems. Conventional management of sweet cherry in Australia, for example, can include use of inorganic fertilisers for nutrition. Integrated pest management involves cultural, biological and varying extents of chemical control, such as the use herbicides for weed control, insecticides for insect pests, fungicides (broad-spectrum or targeted) for disease control and in some cases fumigation has been used to control soil borne pathogens, i.e. *Armillaria* or *Vertiicillium* (Webster et al. 1996). In contrast, organic farmers modify their management practices to suit the requirements of organic certifications which include the use of organic fertilisers such as the application of animal manures (e.g. chicken manure), composts, or certified supplements including microbial, bio-dynamic, or plant-based products (Cubison 2009). Chemical controls are limited, with the use of soft soaps, pyrethrum and some copper based sprays such as Bordeaux spray used as a last resort with a strong focus on prevention rather than cure (Cubison 2009). How these differences influence AMF diversity and abundance in a perennial fruit tree system, such as sweet cherry orchards, is unknown.

This project aimed to study the influence of the management practise on the abundance and diversity of AMF in cherry orchards. The study compared an organic cherry orchard and a conventionally managed cherry orchard in close proximity in southern Tasmania. It was expected that there would be significant differences in abundance and diversity of species of AMF found at the two orchards. In addition, abundance and diversity of AMF was expected to differ between the two seasons.

3.2. Materials and methods

To better understand the role of AMF in sweet cherry orchards, preliminary studies were conducted to assess diversity and abundance in two orchards with different management practices (conventional and organic) in southern Tasmania at two different times of year. AMF diversity was assessed based on spore characteristics and a discriminant analysis was

conducted to explore the impact of site and season on spore diversity. Due to time and funding restrictions, identity of AMF spores was only tentatively determined for major spore types based on morphology.

3.2.1. Field sites

Both orchards included in this study were in the Huon Valley, Tasmania, 800 m distance apart, on undifferentiated alluvial soil on Quaternary alluvium (Musk et al. 2000). According to Musk et al. (2000), soil type is considered to be similar at the two sites. The organic orchard is situated in Grove (GPS coordinates -42.996196, 147.073516) and is planted with 10 year old Mazzard rootstock with Lapins and Simone cultivar scions. The orchard converted from conventional to organic practices 6 years prior to our study. Fertilisation of the orchard includes primarily chicken manure, while disease and pest control relies on sulphur, copper, lime and organic oils. The conventional orchard is situated in Lucaston (GPS coordinates -42.992837, 147.060469) and was planted with approximately 10 years old Mazzard rootstock with Lapins cultivar scions. Conventional fertilizers and pesticides are used routinely. Spacing in both orchards is 4 m row spacing and 1 m tree spacing. Both orchards are irrigated with micro-sprinklers at the tree line.

3.2.2. Soil sampling

Soil was sampled at both orchards in autumn 2013 (March , mean min/max temperatures 8.0 /20.5 ^{O}C) and again in spring 2013 (November , mean min/max temperatures 7.1/18.6 ^{O}C) (*Bureau of Meteorology* 2014). 20 samples of a minimum 4kg of soil taken from around the root zone to 20 cm depth were randomly collected from each site. Samples were stored in plastic bags at 4°C for up to 12 months before processing. Additionally, another 20 samples of a minimum 1 kg of soil were collected from the organic orchard for the pot-trial outlined in chapter 4.

3.2.3. Spores extraction and counting

Spores were extracted from the soil samples from both sites and both seasons, using a sucrose centrifugation method (Sasvári et al. 2012; Sekoele 2006). A 100 g sub-sample of soil was placed in a beaker with 500 ml of water and the soil was brought into suspension by stirring.

After allowing the large soil particles to settle, the suspension was poured through a 710 µm mesh sieve followed by a 45 µm mesh sieve. The soil which collected in the 45 µm mesh sieve was transferred to a beaker and then divided to several centrifuge tubes. A 25 ml volume of 70% sucrose solution was added to each tube, which were then centrifuged at 2000 rpm for 3 min. The supernatant was poured into a 45 µm sieve and washed with tap water. Then spores (with water) were poured into 20 ml tubes. After that, spores were counted from a 1 ml sub-sample from each tube using a Leica stereomicroscope (35× magnification), and this was repeated three times. Spore abundance was expressed as the number of AMF spores per gram fresh soil.

3.2.4. Morphological analysis

Spores from 20 samples were examined under a compound microscope at 400× magnification. Spore characteristics were selected according to the literature (Blaszkowskl 2012; INVAM 2014), and included three categorical variables (shape, colour, pattern) and two continuous variables (spore diameter and the number of spore wall number of layers). Categories for spore shape, colour and pattern were determined from a preliminary assessment of representative spores.

In addition to categorisation, an attempt was made at species identification of the dominant spore types. This was done based on the spore characteristics alone. The morphology of AMF families and species is outlined by INVAM (2014) and includes colour, shape, and spore diameter, as well as spore wall diameter and number of layers in some cases. Pattern was included as spores with transparent layers indicate an outer layer which is soft, while spores with spotted patterns indicate a rigid outer layer. Spores from samples in both sites and seasons were photographed and measured. After that, similar morphologies were grouped together to link them with species of best fit to INVAM descriptions (INVAM 2014).

3.2.5. Statistical analysis

This study aimed to understand the diversity of AMF spores in different sites (organic and conversional soil) in two different seasonal (autumn and spring). Spore count data was therefore analysed with a Generalised Linear Model assuming a Negative Binomial

distribution. SAS version 9.3 was used for canonical discriminate analysis to classify observations into groups (site and season) on the basis of the chosen variables.

3.3. Results

3.3.1. Abundance of spores

The number of spores was not significantly different in terms of seasons (spring and autumn) (P = 0.07). However, the average numbers of spores in the conventional orchard soil was significantly higher (over double) than in the organic site in both seasons (P = 0.0001) (spring and autumn) (Figure 3.1).

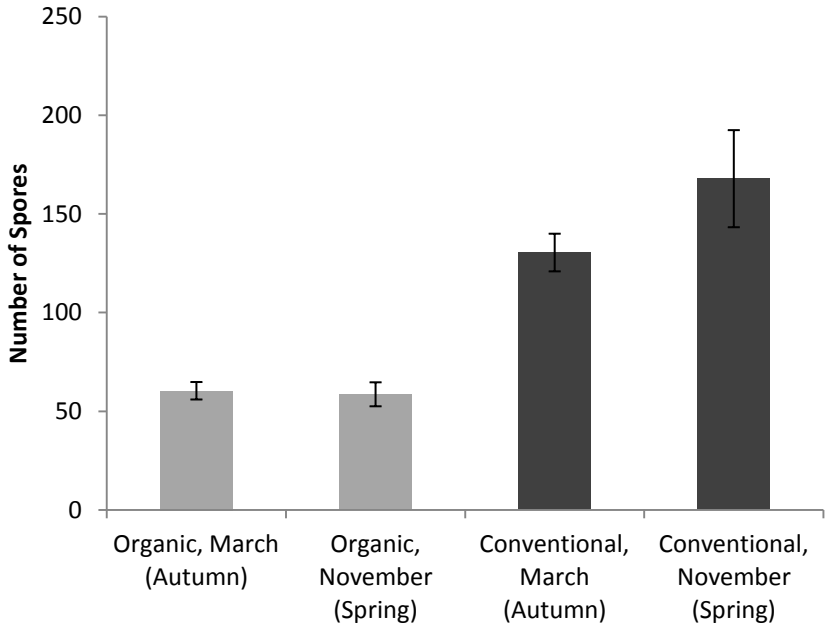

Figure 3.1: Mean number of spores (± SE) in soil samples collected in March & November in organic & conventional cherry orchard soils n=10.

3.3.2. Discriminant analysis of spore characteristics

Canonical discriminant analysis was conducted to determine which linear combinations of spore variables were able to provide maximal separation of the two factors (site and season), or outcome groups. This linear combination achieves the highest possible multiple correlation within outcome groups. The maximal multiple correlations for the first group is called the first canonical correlation. The coefficients of the linear combination are the canonical coefficients or canonical weights. A multivariate analysis of variance indicates that the mean vectors of the groups do differ significantly (Table 3.1).

Table 3.1: Multivariate Statistics and F Approximations S=3 M=5 N=190.5, NOTE: F Statistic for Roy's Greatest Root is an upper bound.

Statistic	Value	F Value	Num DF	Den DF	Pr > F
Wilks' Lambda	0.79658714	2.16	42	1136.9	<.0001
Pillai's Trace	0.21795554	2.15	42	1155	<.0001
Hotelling-Lawley Trace	0.23747840	2.96	42	926.95	<.0001
Roy's Greatest Root	0.10770251	2.96	14	385	0.0003

The canonical discriminant analysis shows that two dimensions have significant P values (Table 3.2). Dimension 1 has a canonical correlation of 0.31, which means it explains 31% of the variation in the data set. Dimension 2 has a canonical correlation of 0.29, so it explains 29% of the variation. The two linear combinations are shown below after standardising the input data variables (subtract the means and divided by standard deviation). The first dimension depends mostly on the colour red and diameter (Table 3, Figure 2) while the second dimension depends mostly on colours red and orange, number of layers, pattern Sp, shape, El, and thickness (Table 3.3, Figure 3.3).

Table 3.2: Canonical discriminant analysis of spore data, showing statistics for the first three dimensions, Analysis was conducted using the CADISC procedure in SAS,

Dimension	Canonical correlation	Approx. Standard Error	Squared canonical correlation	Eigen value	difference	proportion	Cumulative	Likelihood ratio	Approx. F Value	Num DF	Den DF	Pr > F
1	0.311	0.045	0.097	0.107	0.016	0.453	0.453	0.796	2,16	42	1136.9	<0.0001
2	0.289	0.045	0.083	0.012	0.091	0.052	0.383	0.837	1.91	26	768	0.004
3	0.192	0.482	0.037	0.038		0.162	1.000	0.962	1.24	12	385	0.253

Table 3.3: Standardised canonical coefficients for the levels of the categorical variables dimension such as shape, colour, Pattern, transparent, diameter, thickness and Number of layers.

Variable*	Dimension 1	Dimension 2	Dimension 3
Shape SE1	-0.123	0.421	0.359
Shape SG1	0.380	0.211	-0.142
Shape SIr	0.69	-0.003	-0.081
Shape SSu	0.000	0.000	0.000
Colour CB1	0.096	0.062	0.243
Colour CBr	0.118	0.231	0.384
Colour CGr	-0.309	-0.145	-0.024
Colour COr	-0.321	-0.145	-0.024
Colour CRe	-0.573	-0.179	-0.218
Colour CWh	-0.039	-0.190	0.379
Colour CYe	0.000	0.000	0.000
Pattern PSp	0.149	0.399	-0.439
Pattern PSq	0.371	-0.169	-0.149
Pattern PTr	0.000	0.000	0.000
diameter	-0.369	-0.126	-0.177
thickness	-0.145	0.540	0.177
Number of layers	0.620	-0.5449	0.073

*SG1: Shape global, SSu: Shape sub global, SE1: Shape ellipsoid, Sir: Shape irregular, CB1: Colour black, PSp: Pattern spots, PSq: Pattern squared, PTr: transparent, CBr: Colour brown, CGR: Colour gray, COr: Colour orange, CRe: Colour red, CWh: Colour white, CYe: Colour yellow.

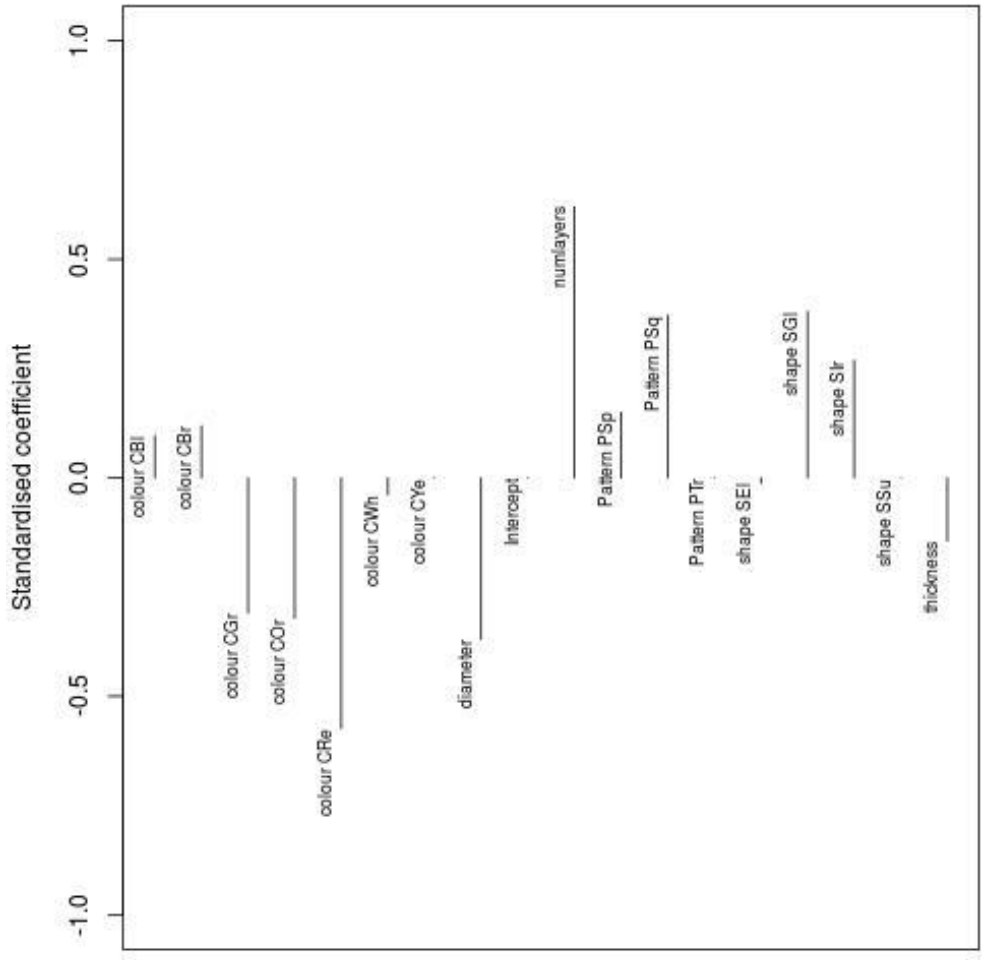

Figure 3.2: Canonical discriminant variables for dimension one using standardised data.

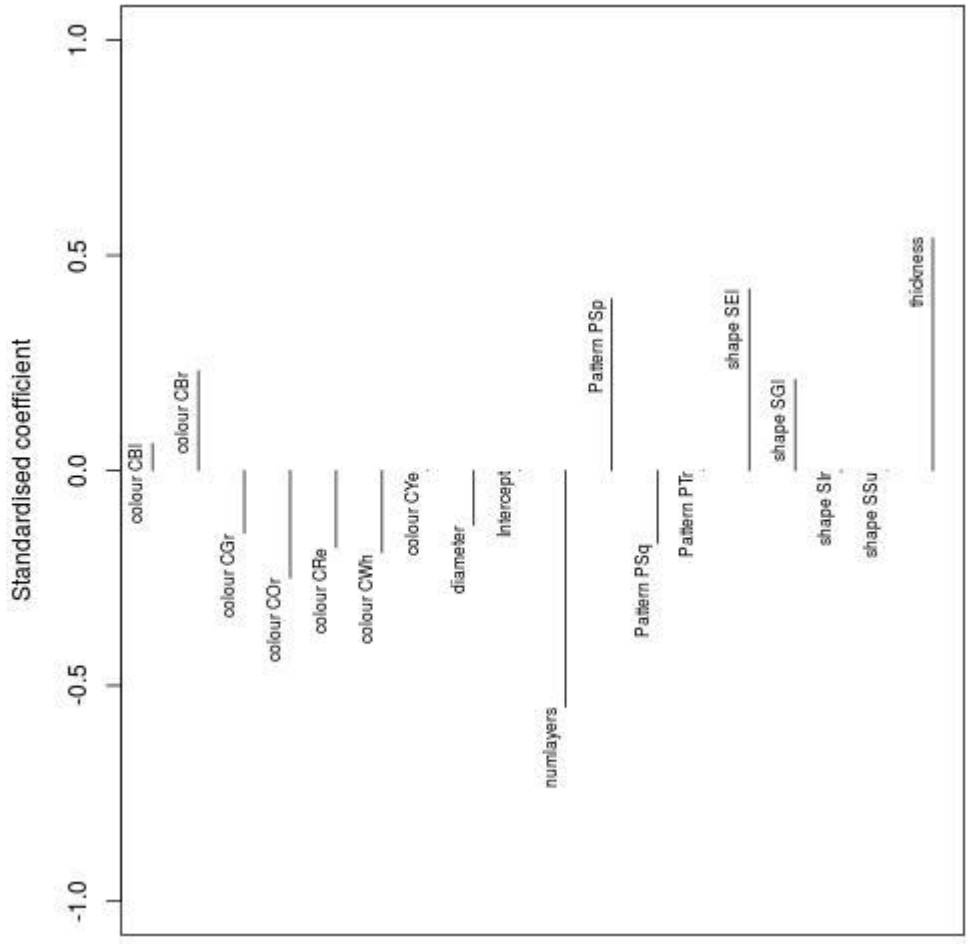

Figure 3.3: Canonical discriminant variables for dimension two using standardised data

Plotting the two dimensions for each outcome group (for site and season) resulted in considerable overlap suggesting there is a high level of similarity in spore characteristics (Figure 3.4). However, the mean of the `Nov-S1' outcome group is slightly offset on dimension 1 while March-S2 is offset on dimension 2 (Figure 3.4). Therefore, while small, the biggest difference in diversity is between different sites in different seasons.

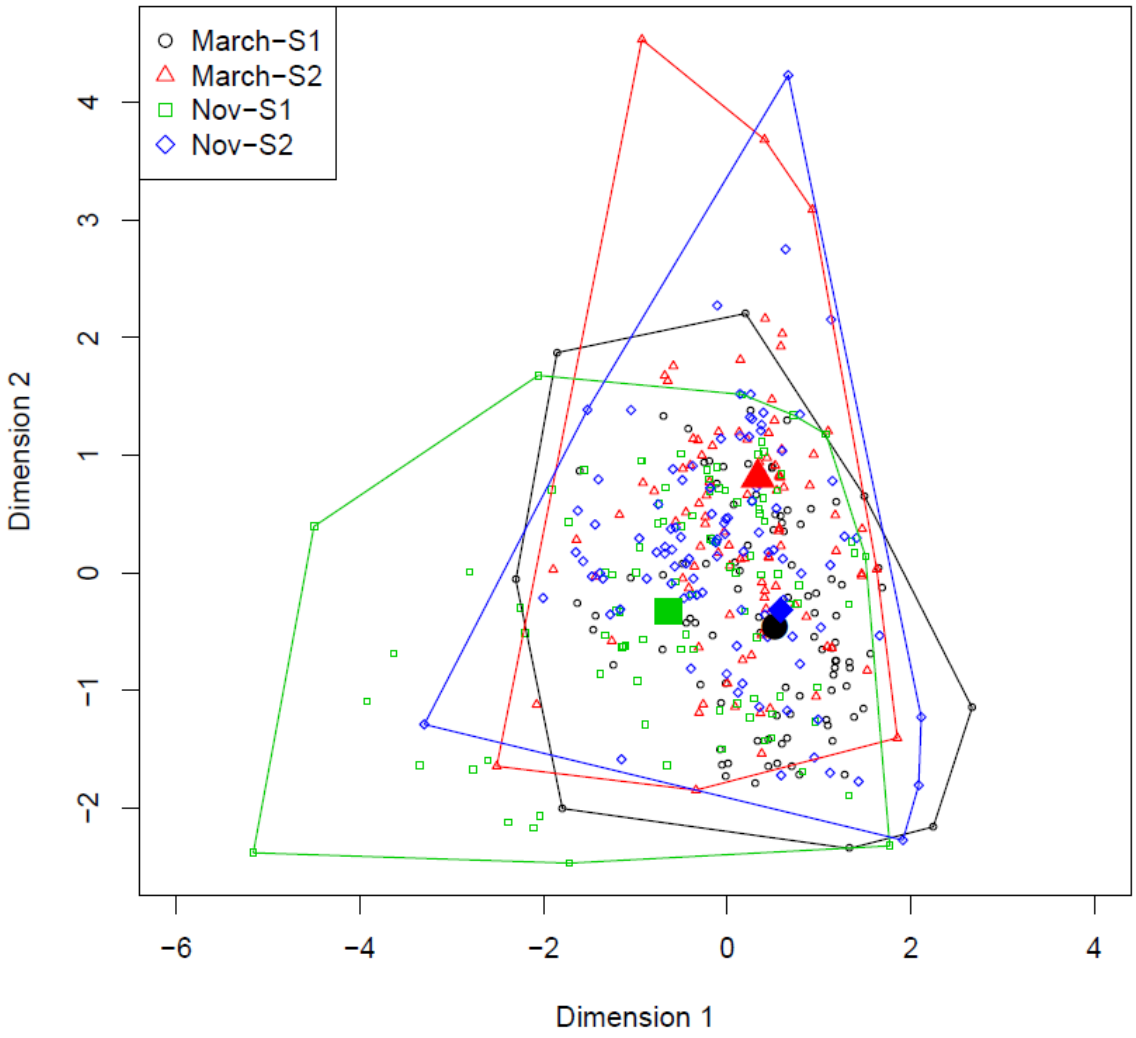

Figure 3.4: Canonical discriminant plot of the first two dimensions, with means for each outcome group also shown (indicated by larger markers).

3.3.3. Typical spore characteristics

Examples of the most typical AMF spores found in both sites (organic and conventional cherry orchard soils) are shown in Figure 3.5.

Type 1 (Figure 3.5a): Spore colour from red-brown to almost black, and shape globose to subglobose, rarely irregular (Possible identity *Septoglomus constrictum*).

Type 2 (Figure 3.5b): Spores coloured pale orange-brown to dark orange-brown, with most orange brown in the field or after long storage, and shaped among globose to subglobose, rarely irregular. Size diameter: 80-220 µm (INVAM 2014) (Possible identity *Funneliformis coronatum)*

Type 3 (Figure 3.5c): Spores that had similar colour were grouped according to INVAM: yellow brown, sometimes with a green tint. Size diameter: 40-140 µm, Layer 2 (L2): Adherent to the mucilaginous outer layer, hyaline, 1.5-4.9 µm thick (Possible identity *Glomeraceae, Rhizophagus intraradices*). Although one of the spores had outer layer of 5.4 µm diameter, due to other properties it may fit in this group.

Type 4 (Figure 3.5d): Colour: Bright yellowish orange, Shape: Globose, subglobose, occasionally ovoid, diameter size: 100-240 µm (INVAM 2014) (Possible identity *Funneliformis verruculosum).*

Type 5 (Figure 3.5e): colour pale yellow to pale brownish-yellow; shape: globose, subglobose, ellipsoid, pyriform, or irregular, often lenticular, size diameter: (60-) 121 (-130) x (70-)118 (-200) µm. spore wall consists of three layers (L1, L2, and L3). The outer layer (L1) is rigid, permanent, and consists of several sublayers (laminae), pale yellow to yellow-brown in colour, 0.8-2.5 (-3.5) µm thick (INVAM 2014) (Possible identity *Diversisporaceae, Diversispora trimurales*).

Type 6 (Figure 3.5f): colour light brown; shape globose, subglobose, ellipsoidal or irregular size diameter: (50-) 80-120 (-155) x (45-) 75-120 (-140) µm. The spore wall is composed of two distinct, separable layers (L1 and L2). The outer layer (L1) is light yellow to orange-yellow (2-) 4-6 (-8) µm thick (INVAM 2014), so possible identity *Glomus hoi*).

Type 7 (Figure 3.5g): colour pale yellow with a greenish tint to yellow-brown with greenish tint in older spores; shape wide range, from subglobose to ellipsoid to oblong, sometimes irregular; diameter size: 120-220 µm *(*Possible identity *Scutellospora calospora).*

Type 8 (Figure 3.5h): colour bright white to pale cream, shape most often tear-drop shaped to irregular, more rarely globose, subglobose; size diameter: 40-60×140-160 µm (globose spores 40-120 µm (INVAM 2014), (Possible identity *Diversisporaceae, Diversispora eburnean*).

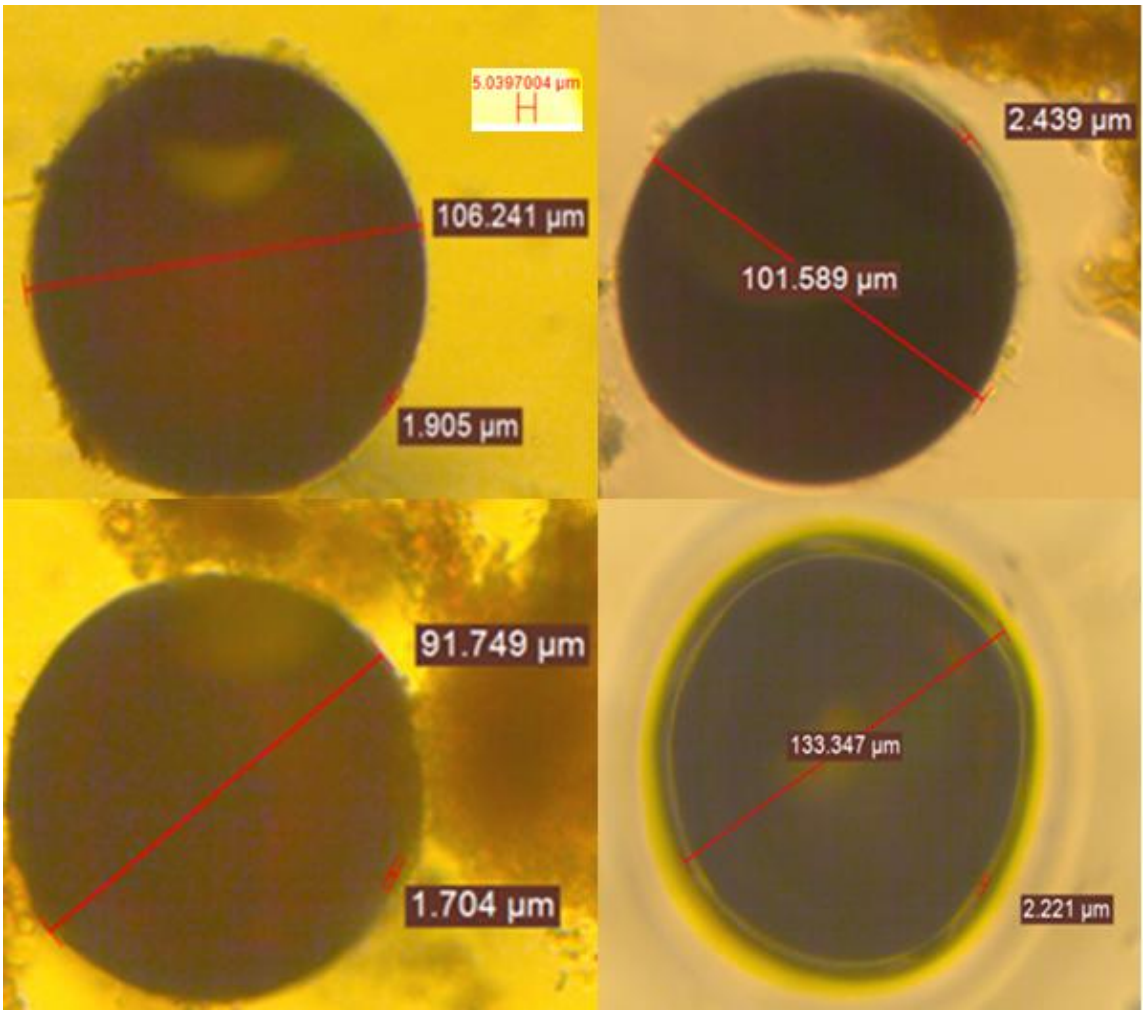

Figure 3.5a: AMF spore type 1; possible identity *Septoglomus constrictum.*

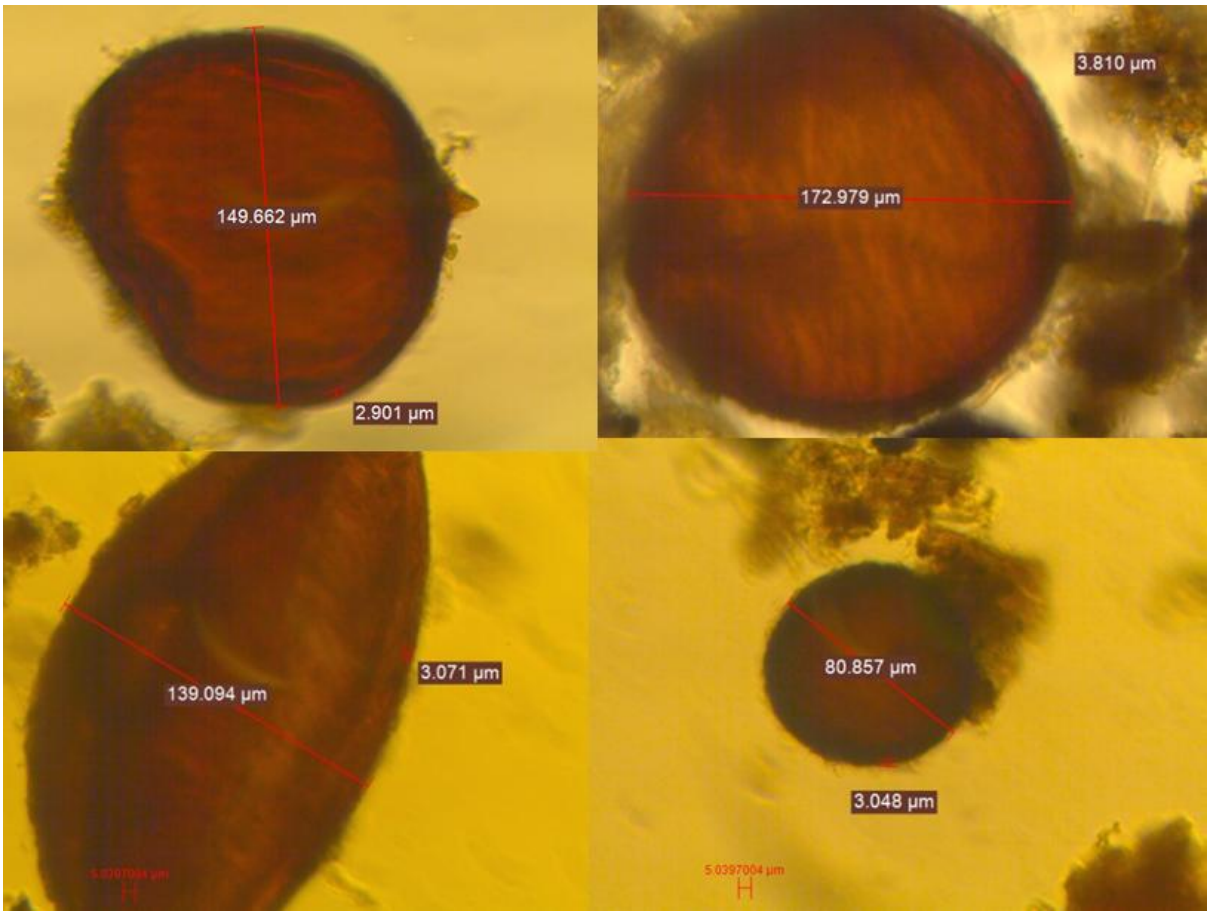

Figure 3.5b: AMF spore type 2; Possible identity *Funneliformis coronatum.*

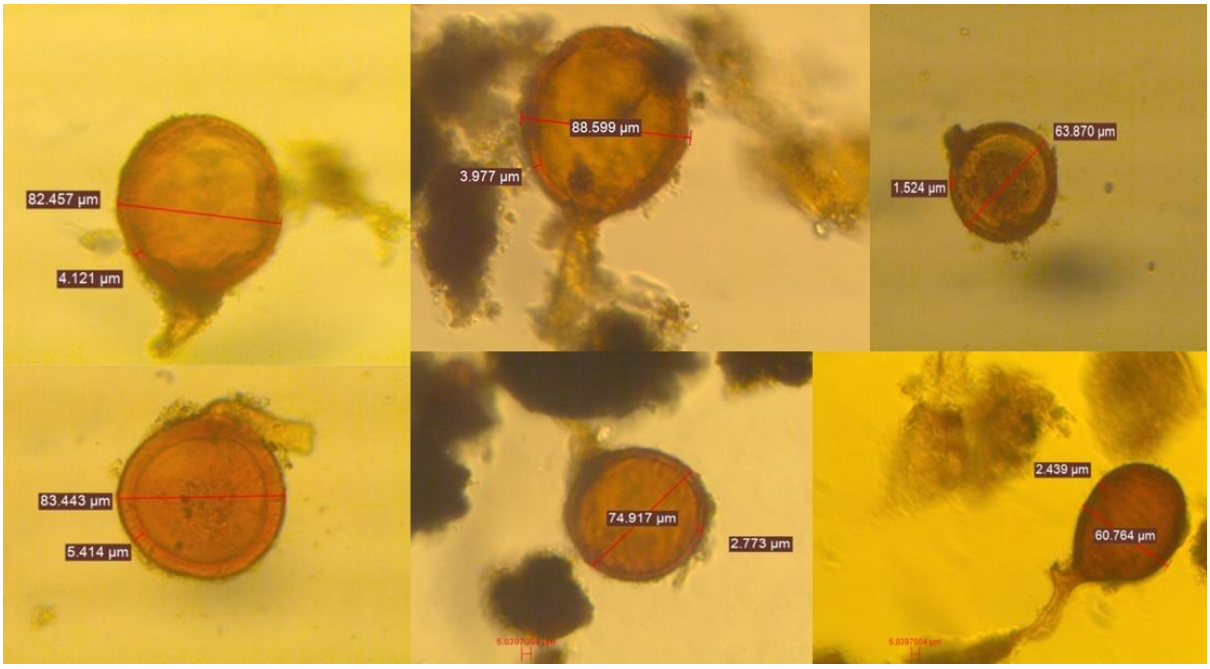

Figure 3.5c: AMF spore type 3; Possible identity *Glomeraceae*, *Rhizophagus intraradices.*

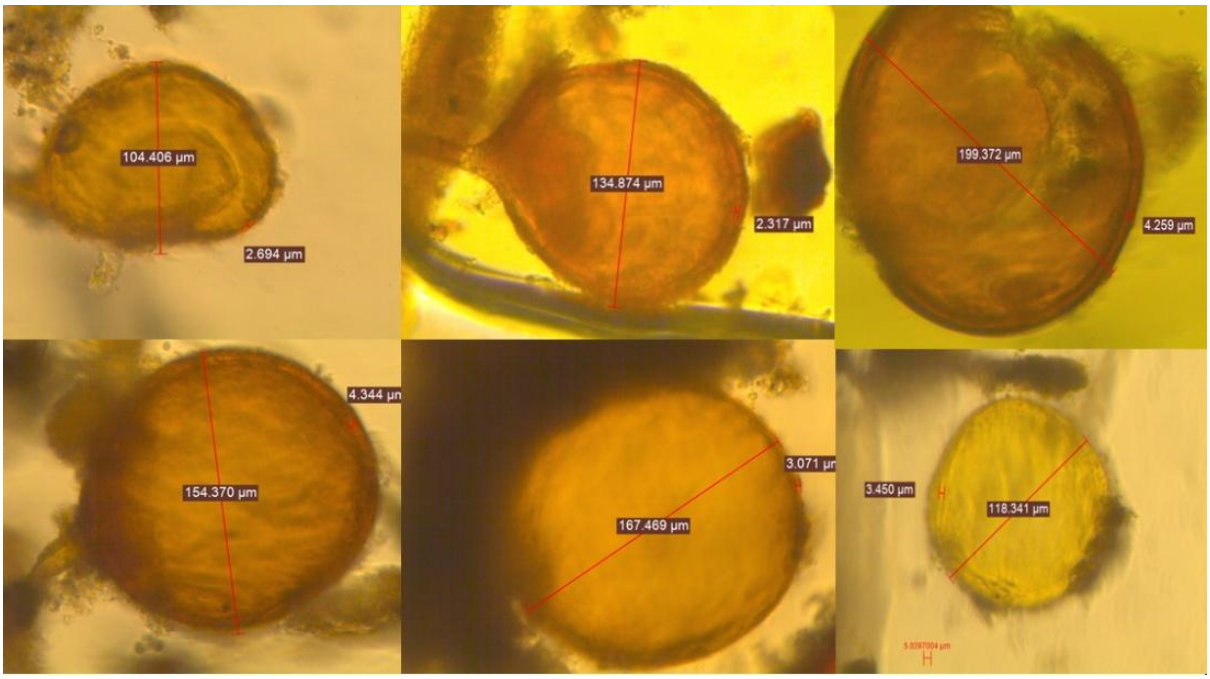

Figure 3.5d: AMF spore type 4; Possible identity *Funneliformis verruculosum.*

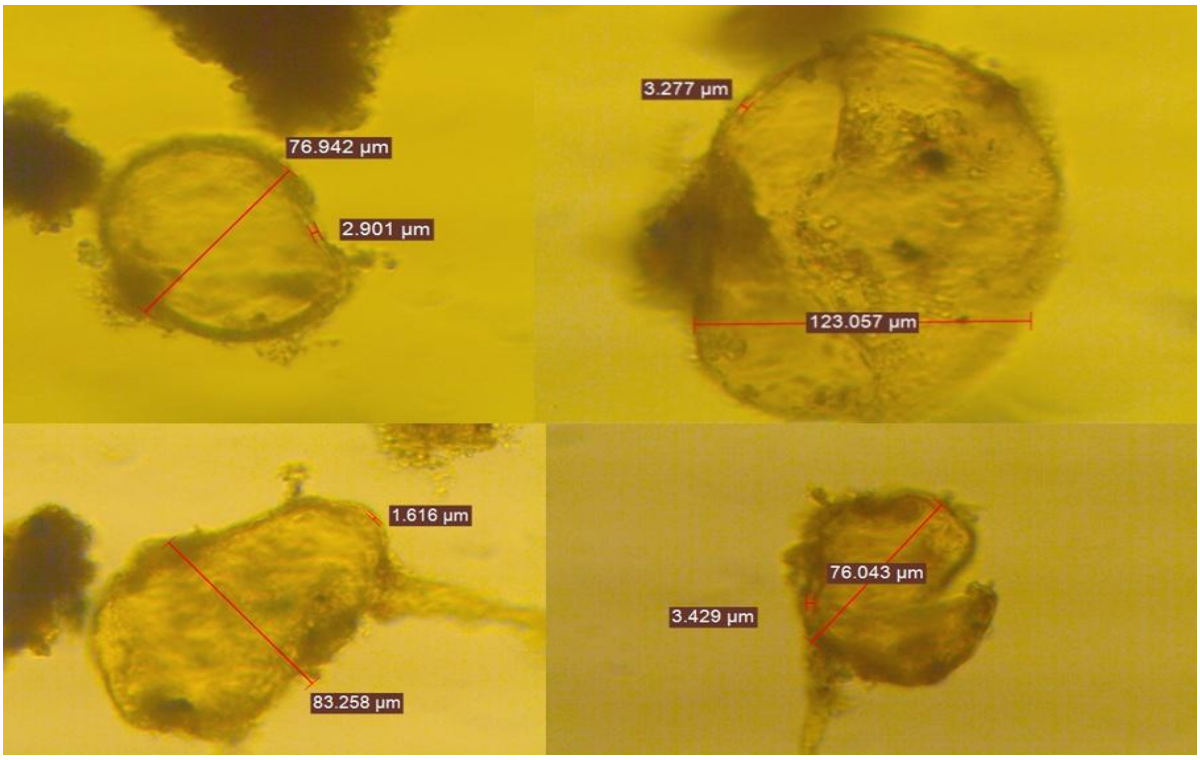

Figure 3.5e: AMF spore type 5; possible identity *Diversisporaceae, Diversispora trimurales.*

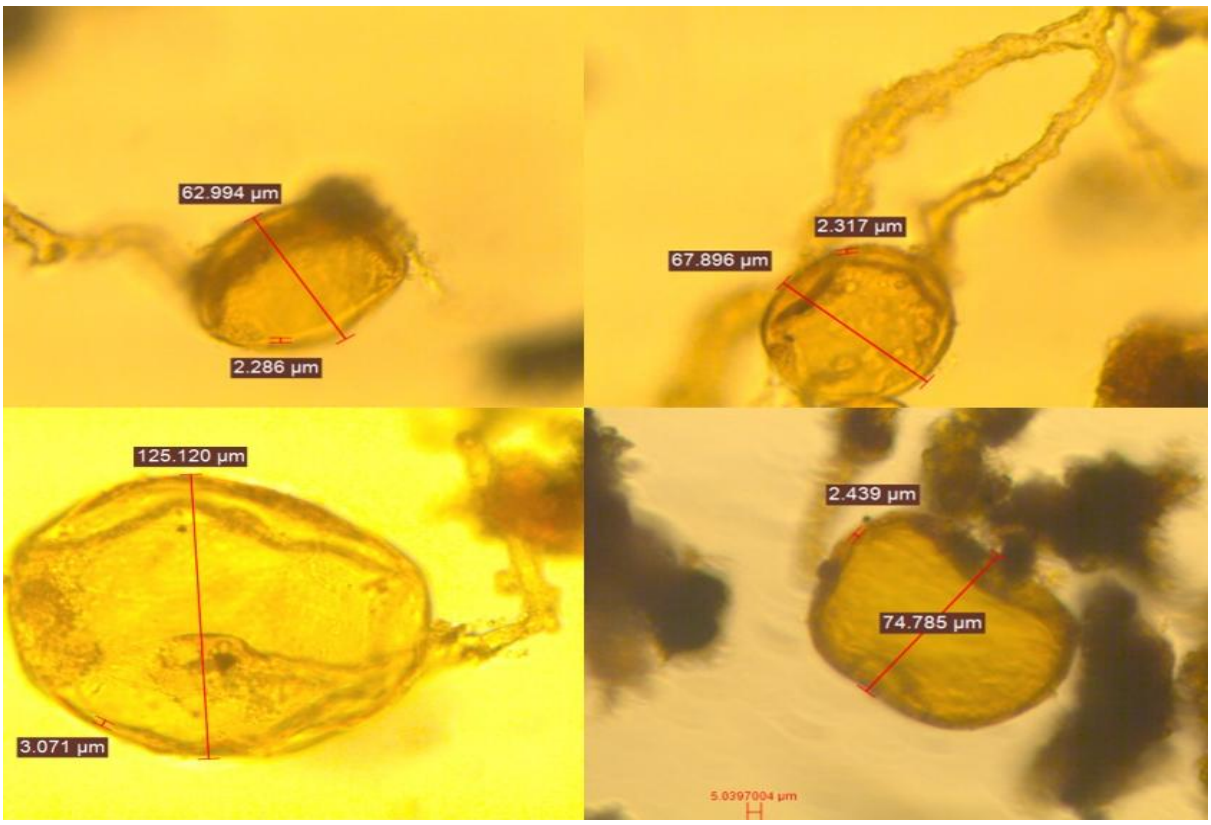

Figure 3.5f: AMF spore type 6; possible identity *Glomus hoi*.

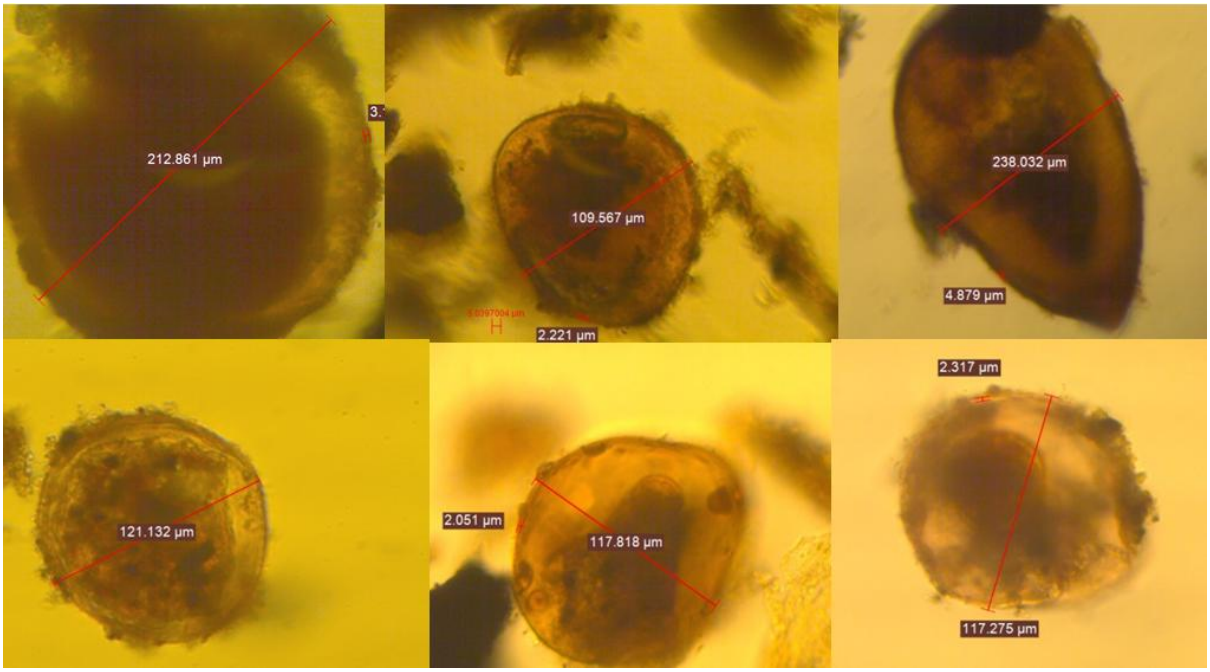

Figure 3.5g: AMF spore type 7; possible identity *Scutellospora calospora.*

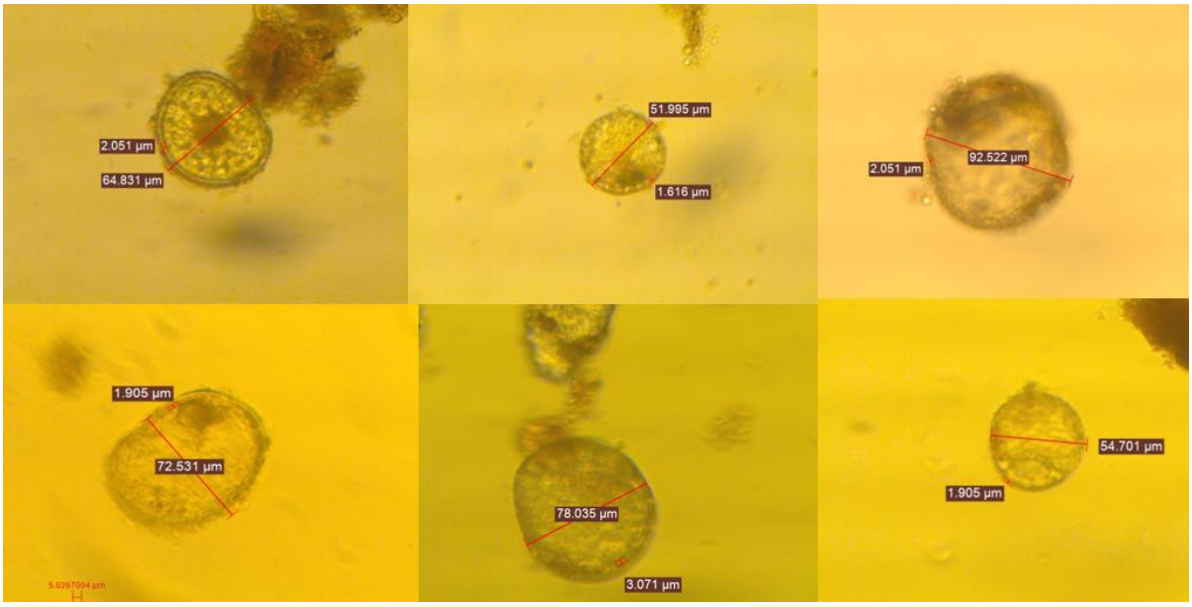

Figure 3.5h: AMF spore type 8; possible identity *Diversisporaceae, Diversispora eburnean.*

3.4. Discussion

3.4.1. Effect of site and season on spore counts

The effect of different sites (organic and conventional cherry orchards) on abundance of spores was significant. However, the abundance of spores recorded in the conventional site was significantly higher than in the organic soil site in both seasons (March, November). This was not expected based on a previous research by Oehl et al. (2004) who did a perennial system study on grass-clover meadow over 2.5 years and found that inorganic fertilisers limited the development of AMF colonisation, whereas organic fertiliser promoted them.

It has been recorded that the lifetime of arbuscules is shorter than the host cell (around 8.5 days) (Parniske 2008). As a result, the spores and the hyphae in root fragments are the main source of infection at the initiation of the growing season (McGee et al. 1997). While weed cover was not quantified, it may be that the higher abundance of spores in the conventional orchard could be due to more herbaceous weeds and grass around the cherry trees than in organic orchard, which agreed with Dai et al. (2013) and Oehl et al. (2004) who found that abundance of AMF in native grassland was higher than in cropland. In addition, we think that inoculum viability may be affected by storage of AM fungal propagules in soil at 4°C. However, few data are available on the survival of spores over prolonged periods of storage under various soil moisture conditions (Ruiz-Lozano et al. 1996).

Spores isolated using sucrose centrifugation do not fully represent all AMF likely to be present at the sampling point. That is, it would only include fungi that produce external spores, not those which formed spores in roots alone or in sporocarps (An et al. 1990). Additional drawbacks include the fact that the larger spores of some AMF species (e.g. Gigaspora calospora) spores may sink along with soil particles during the decantation step of the wet-sieve method (Smith et al. 1979). However, Brundrett et al. (1996) stated that spores isolated by centrifuged sucrose solution at 2000 rpm are relatively free of other debris and any remaining organic from denser soil components. For these reasons, the wet-sieve method is recommended for the collection of spores when large numbers are desired and to compare numbers in sparsely populated soils as might be encountered in field samples (Smith et al.

1979). In general, Cardoso et al. (2003), found the number of spores was ranging on average from 2 to 130 g−1 soil, and Proborini et al. (2013) found that the average of spores ranged between 52 to 91 per 250 gram. It is possible that spores of other fungi that are within the sieving mesh size (45 µm to 710 µm) were present in our samples of isolated spores. While the majority of soil fungi (e.g. Penicillium species) produce very small spores which would have been excluded from our samples, some other less abundant species produce larger sexual resting spores (e.g. some ascomycetes and basidiomycetes). We were confident in our assessment of spores as AMF based on the size and morphology.

Chaturvedi et al. (2012) and Lee et al. (1994) found that AMF spore abundance was affected by season. For example, in autumn the number of spores increased as a result of sugar allocated in roots and rhizomes, compared to spring where more photosynthate was allocated to flower development. However, the present study found different seasons did not significantly impact on abundance of AMF spores in either site, despite a slightly increased number of spores in conventional orchard soil in spring (November) than in autumn (March). Therefore hese finding were not in accordance with the study reported by Gould et al. (1996) who stated the maximum root colonisation and spore population density occurred during the early summer season and the minimum during autumn on reclaimed sites in Kentucky. A study by Hijri et al. (2006) based on molecular evidence found that AMF were more active in spring than in autumn. This did not agree with our result. It could be as a result of close temperature in both seasons.

3.4.2. Effect of site and season on spore diversity

Our result showed that there is a high level of similarity in spore characteristics (Figure 4), so we expect similar spores in both sites. However, studies by several authors (Bainard et al. 2011; Lee et al. 2009; Oehl et al. 2004) found that conventional practices effect community compensation of AMF, and the spore abundance and diversity of AMF were significantly higher in organic management than in conventional soil. Daniell et al. (2001) stated that the level of spore production does not reflect the abundance of species in roots. Chaturvedi et al. (2012) stated that in autumn *Glomus claroideum, Acaulospora scrobiculata* and *A. spinosa* were found at all sites, while in spring only *G. intraradices* was found, but was found everywhere (an arable field, oak forest etc). Also Dai et al. (2013) found that soil types have

the biggest effect on AMF communities rather than crop management. In this study we found a similar diversity of AMF spore morphology in different sites that have same soil type.

Although Hijri et al. (2006) concluded that low inputs are needed to maintain AMF diversity, they stated that it is not easy to correlate between a high diversity of AMF in organic management. Hijri et al. (2006) found low AMF diversity in organic management comparing with conventional management, but their organic soil history had high phosphate input. . Methods to assess AMF diversity and abundance are challenging, given that these fungi are obligate biotrophs and cannot be cultured. Traditional identification is based on morphology and relies on spore characteristics (Schenck et al. 1990), but recent methods also include features of arbuscles present in the colonised roots (Blaszkowskl 2012). For this reason, an ideal way to identify AMF using traditional methods is to prepare single-spore trap cultures (Jansa et al. 2002; Redecker et al. 2003), so that spores can be easily matched with hyphae in colonised roots. In the past decade use of molecular techniques have become widespread for identification of spores (Silva et al. 2012) or community analysis (Bainard et al. 2014). A recent review outlines that the molecular diversity of Glomeromycota fungi can be reflected using molecular operational taxonomic units (MOTU's) in the absence of species identification (Öpik et al. 2013). These approaches were beyond the scope of our study but would be worthy to pursue in future research.

3.5. Conclusion

This study provides only a preliminary characterisation of AMF abundance and diversity. Although the organic cherry orchard soil had smaller numbers of AMF spores compared with conventional cherry orchard soil, similar numbers were maintained during different seasons (autumn and spring). The higher abundance of spores in the conventional orchard could be due to more herbaceous weeds and grass around the cherry trees than in organic orchard. A high level of similarity in spore characteristics, so we expect similar spores in both sites. That roots were not examined means that the results presented here are based more on those species that are prolific sporulating species. Future studies could include generation of specific spores in trap culture and could also further assist in terms of identifying spores. It became clear that certain AMF species could not be confidently identified in the field samples; the morphological features of the spores were not distinct enough, thus use of

molecular analysis to link the spore morphology analyses with their genus or species would also be ideal. Better knowledge of diversity of beneficial microbes in soil such as AMF could lead to better management of this poorly understood resource.

Chapter 4 Effect of AMF on Establishment and Water Relations of Sweet Cherry Rootstocks *(Prunus avium L* x *Prunus pseudocerasus)*

4.1. Introduction

There are two main concerns currently facing cherry growers related to water availability: fruit cracking and the adverse effects of drought. If cherry trees are exposed to excess water, particularly heavy rainfall late in the growing season when the fruit is maturing, the fruit can suffer from cracking, which causes a decline in quality (Balbontín et al. 2013; James et al. 2011; Measham et al. 2012; Simon 2006). Recent studies in sweet cherries have demonstrated that cracking in sweet cherry results from both water flow across the fruit cuticle from surface water and flow from the soil through the vascular system. This can result in downgraded fruit quality or an unsaleable crop (Measham et al. 2014). Fruit cracking can be mitigated by increasing skin plasticity through regulating water (James et al. 2011). Skin plasticity can be increased by water management which provides continuous water uptake and avoids water deficit followed by water excess (Measham et al. 2014). According to Birhane et al. (2012) and Quilambo (2004b), drought conditions negatively impact on plant functions by inhibiting photosynthesis and disturbing the delicate mechanisms which regulate oxygen levels in the nodules. Drought can have significant effects on cherry yields, size, and production times (Bright et al. 2004).

As AMF can assist host plants to increase water uptake (Asrar et al. 2012; Augé 2001), there is the possibility that mycorrhizal cherry trees may be less prone to fruit cracking than non-mycorrhizal cherry trees. According to a number of studies, arbuscular mycorrhizal fungi (AMF) can control water in both extremes of water availability: drought and abundant water conditions (Augé 2001; Birhane et al. 2012; Koltai et al. 2010b)**.** It has been shown that inoculated plants, when subjected to drought conditions, have higher transpiration and stomatal conductance, as opposed to non-inoculated plants which maintain closed stomata to avoid drought (Borkowska 2002; Subramanian et al. 2006). Fini et al. (2011) demonstrate that AMF can affect the morphology of the roots of inoculated plants by increasing root growth, amplifying the amount of water accessible in the soil. Furthermore, since mycorrhizal symbiosis can establish naturally in the soil and assist plants to respond to low water conditions by scavenging water more effectively, then AMF symbiosis could

maintain water balance and assist the plant to open their stomata to improve carbon gain under drought conditions (Augé 2004; Fini et al. 2011).

The association between AMF and host plants occurs in most fruit tree species and may become established naturally in the nursery or when transplanted into an orchard or garden (Calvet et al. 2004). Inoculation of stone fruit such as peach with the AMF species leads to increased capacity for rapid plant growth (Rutto et al. 2002). Calvet et al. (2004) point out that plants colonized with *G. intraradices* today known as *'Rhizophagous irregularis',* have a higher rate of root infection than other *Glomus spp*. In Tasmania, there is currently no information about which mycorrhizal fungi are dominant in most agricultural or horticultural landscapes, including sweet cherry. Although it is expected that *R. irregularis* is present in Tasmanian soils, given its ubiquitous distribution globally, it may not be the most common AMF species associating with sweet cherry plants in southern Tasmania. For this reason, it is relevant to use locally abundant species in studies which have implications for practical management. Field soil can be used to provide local AMF inoculum which provides a more realistic (although less controlled) scenario. Ideally local AMF species would be individually isolated in pot-cultures and identified in association with any studies.

There were multiple purposes of the studies presented in this chapter, including to determine the influence of AMF (both *R. irregularis* and local AMF species) on sweet cherry rootstock related to survival of cuttings, and to investigate how AMF effect water uptake for growth and physiological functioning in both drought and excess water conditions.

Experiment 1:

Aim: To investigate the effect of *R. irregularis* colonisation on establishment of sweet cherry from cuttings.

Hypothesis: It was hypothesized that sweet cherry plants would have greater survival and establish more rapidly from cuttings inoculated with AMF than those not inoculated (Gosling et al. 2006; Krishna et al. 2005; Mukerji et al. 2000; Rutto et al. 2002).

Experiment 2:

Aim*:* To investigate the effect of *R. irregularis* colonisation on sweet cherry growth and physiological response to drought and excess water

Hypothesis: Under different water conditions we hypothesized that inoculated plants would have considerably higher water absorption potential, photosynthetic rate (CO_2 uptake) and stomatal conductance than their non-mycorrhizal counterparts. Additionally, it was expected that cherry rootstocks colonized by AMF would have faster growth in terms of number of leaves, stem diameter and biomass, compared with non-inoculated plants .

Experiment 3:

Aim: To investigate the effect of colonisation by local AMF species on sweet cherry growth and physiological response to drought and excess water.

Hypothesis: That filtering field soil to remove spores would result in significantly less AMF colonisation of sweet cherry than unfiltered soil. If significant differences in colonisation were achieved, the same hypotheses as for experiment 2 would be posed.

Experiment 2 and 3 included root stock which was inoculated with AMF in pot trials and then subject to different water regimes. A variety of measurements were undertaken to examine plant growth and water relations, including gas exchange, water potential and plant hormone analysis of plant tissue. This will provide an indication of the effect of AMF on plant biomass, growth, photosynthetic capacity, and water uptake and stress of plants growing under different environmental conditions. Therefore, experiments were designed to explore water regulation of mycorrhizal cherry rootstocks which may lead to improvements in fruit quality as a result of improved plant function, as well as enhanced growth performance and nutrient uptake.

4.2. Materials and Methods

Three separate experiments were conducted in Hobart, Southern Tasmania to support the above objectives. In 2012-13, the first experiment was undertaken from November 2012 to

April 2013, and the second and the third experiments were undertaken, simultaneously, from August 2013 to March 2014.

4.2.1. Plant material

For all experiments, sweet cherry rootstock "Colt" (*Prunus avium* x *Prunus pseudocerasus*) was obtained from the nursery of a commercial orchard. The dormant rootstock was maintained in clean soil (steam sterilised vermiculite and peat soil) and kept moist at 10 ^{o}C until planting. Cherry cuttings of about 20-30 cm length were prepared and dipped in Indole Butyric Acid (IBA) rooting powder (Rhizopon, Hortus USA) (Hansen et al. 1968), followed by a treatment of AMF inoculum or control (see section 4.2.4.). They were then planted into sterilized 2 inch plastic pots, containing a sphagnum peat moss from Lithuania, pH= 3.5-4.5 (Klasmann- Deilmann GmbH, Germany). and coarse (grade 4) perlite (Exfoliators Aust Pty Ltd, Australia) growing medium (1:1) that had been steam sterilized on the morning off set-up. The pots were then placed on benches bottom heated up to 20 ^{o}C with moisture provided by misters and grown for two months before transplanting took place as follows:

- In experiment 1 and 2: rootstock was transplanted into six inch sterilized plastic pots, each containing an equal amount of sphagnum peat- perlite.

- In experiment 3: the cherry rootstock was transplanted into six-inch sterilized pots, each containing a mixture of equal parts field soil and perlite.

4.2.2. Growing conditions

During the experimental periods, all plants were maintained in a glasshouse at mean temperatures between 18- 25 ^{o}C, and with a mean humidity of approximately 67%, and natural light was supplemented by 400W mercury vapor lamps to give 16 hour day-length. Irrigation was supplied by overhead mist-sprinklers apply water at rate of 7 mm/day was applied by 3 irrigation/day x 7 minutes, Fertilizer and pest management differed for each experiment, as below.

- Experiment 1: Osmocote "Exact" 5-6 month controlled release fertiliser, (Scotts, Australia, 11-11-18 +TE) was applied at a rate of 3kg/m^3 in the second week, and no fungicide was applied for the first five months of the experiment.

- Experiment 2 and 3: all cherry rootstock samples were fertilized, once a week from week 5, with 80 ml of a modified Hoagland's solution with very low phosphorus content (0.05 µM NaH2PO4) (Foo et al. 2013). Then, in the remaining weeks, the plants received 100 ml per pot of the same solution, twice a week. In week 12, a fungicide drench was applied to all the cherry rootstock by using Previcur (Bayer CropScience LP, US) at the rate of 15ml/10l. Additionally, in week 10, the anti-black cherry aphid insecticide, Primor Aphicide (Syngenta Crop Protection Pty Limited, NSW, Australia) was also applied.

4.2.3. Experiment design

Experiment 1 was conducted as a randomized complete block design that initially included 120 individual cuttings (60 inoculated, 60 controls). Unfortunately, most plants were lost in week 29 due to severe spider mite infestation. Although these rootstock samples were drenched by miticide spray, the mite populations persisted. The final replicate number remaining at the end of the experiment was 8.

Experiment 2 and 3 were conducted as randomized complete blocks in a 2 x 2 factorial design. Experiment 2 had 8 replicate blocks, making a total of 32 plants (blocked by bench location), while experiment 3 had 5 replicate blocks, making a total of 20. Treatments included two water supply levels (fully irrigated, and water stressed followed by excess water and two inoculum levels (with and without inoculation).

4.2.4. Treatments

4.2.4.1. Fungal material and inoculation

In experiment 1, *Rhizophagus irregularis* (Myke-Pro wp, Premier Tech Pty. Ltd, Quebec, Canada; 500 spores per g in a sand matrix) (M) was added to cuttings by dipping at the time of transplanting. Non-inoculated plants were dipped in control product (Premier Tech Pty. Ltd, Quebec, Canada) that had no *R. irregularis* spores included (C).

In experiment 2, in week 13, plants were inoculated with the same *Rhizophagus irregularis* spore product as above, (but in two ways. Firstly, when plants were transplanted, approximately 500g of inoculated root fragments of spring onion (*Allium fistulosum*) plants,

which had been inoculated approximately 12 weeks earlier with the fungal spores, were added to the potting medium to boost the rate of colonisation. After extracting and cutting the onion roots to fragments, about 500 g of root fragments were added to each cherry pot. In addition, a solution of 0.1 g/ml of inoculum (added 10g of fungal spores diluted in 100 ml of water) was added to each inoculated pot as a 10 ml dose at two and four weeks post - transplant (Foo et al. 2013; Gao et al. 2001). All non-inoculated cherry rootstock received 500g of non- inoculated root fragments of spring onion (*Allium fistulosum*).

In experiment 3, at week 11 cuttings were transplanted to field soil in pots. A wet sieve and decanting method (Jones et al. 2004) was used to attempt to remove spores from field soil. The field soil was collected from an organic cherry orchard in Grove, which has been described in Chapter 2. After wetting and decanting soil by soaking 20 litre of soil overnight and pouring off the suspension to be used as non-inoculated soil, the resultant solution was filtered to 45µm (which should exclude most AMF spores). The filtered solution was returned to the soil to return other soil microbes and particulates. For inoculated soil, after wetting and decanting, the solution was returned to the soil without filtering.

4.2.4.2. Water

Initially, all plants were watered adequately to avoid water stress until growth was well established. Irrigation levels were determined from the water holding capacity of the soil (Gessert 1976; Novák et al. 2006). This was done by weighing pots (with plants) from one experimental block after they had been allowed to dry. Then, the pots were filled with water, then left to drip to reach field capacityfor about an hour and until excess water had stopped dripping from the pots, then they were weighed once more. The water holding capacity was calculated from the dry and wet pot weights, and irrigation to maintain plants without stress was assigned as 100% water holding capacity.

Plants were subject to one of two treatments by hand watered: DW-EW: A period of deficit water treatment (DW) followed by excess water (EW). AW-EW: Control plants received adequate water (AW) during the deficit water period, but they also received excess water (EW) as well. Plates were located under the pots in excess water period.

DW was applied in week 26 for experiments 2 and 3. This treatment consisted of applying irrigation to 50% of the amount of irrigation water that was previously used to reach water holding capacity. DW was imposed for 29 and 23 days for experiments 2 and 3 respectively. The length of this period was determined by regularly monitoring the difference in leaf water potential between DW and AW plants until a substantial (at least 50% reduction in midday leaf water potential \measurements) difference was obtained, after which DW plants received 50% water holding capacity once a week, but AW was watered daily to maintain 100% water holding capacity. In experiments 2 and 3, DW condition and AW was followed by EW by applying irrigation to 150% water holding capacity to plants. Excess water was applied for 4 days in experiment 2 and 3. In experiment 1, DW was imposed for 24 days before losing plants by severe spider mite infestation in week 27, therefore the EW was not applied.

4.2.5. Assessments

4.2.5.1. Root colonisation by AMF

At the end of each experiment, roots were sampled with three soil cores (1.5 cm × 6 cm) which were rinsed free of soil immediately. Root colonisation with AMF at the conclusion of each experiment was estimated after preparation of samples according to the ink and vinegar method (McGonigle et al. 1990; Vierheilig et al. 1998). 1cm lengths of root were washed and boiled at 300 ^{o} C with 10% of KOH for 10 minutes, then rinsed twice with tap water. After that, they were placed in 3.5% of HCL for 2 minutes. The liquid was then decanted through a tea strainer and the roots placed back into the bottle, covered with 5% Schaeffer black ink in vinegar, and boiled for 3 minutes. Finally, they were rinsed once with tap water and covered with distilled water and drops of vinegar to prevent any contamination.

The number of AMF colonising the root cells was quantified by using a compound microscope and cross-hairs eyepiece graticule, with 150 intersections at presence of hyphae arbuscules and vesicles or nothing were recorded (McGonigle et al. 1990).

4.2.5.2. Growth responses

In all experiments, the growth response of plants was assessed regularly by measuring plant height, and stem diameter, at a height of 3 cm above the ground, and recording the number

of fully developed leaves on each rootstock. The biomass of dry leaves, stems, and roots was measured at the conclusion of the experiments.

4.2.5.3. Physiological responses

Physiological assessments were repeated at three intervals in each experiment: "pre-treatment" (time 0), "mid-treatment" (at the completion of the water deficit period), and "end-treatment" (on days 1 and 3 of recovery for experiments 2 and 3). Gas exchange was measured using an infra-red gas analyzer (LI-6400 XT LI-COR Inc., Lincoln, NE) with the midday photosynthetic photon flux density (PPFD set at 1500 µmol m^{-2} s^{-1}. Leaf water potential (LWP) of the top-most fully-expanded leaf was measured using a pressure chamber ((Bar) (Model 615 PMS Instrument Company, Albany, USA) (Smith et al. 2003).

4.2.5.4. Abscission Acid (ABA) Extraction, Purification, and Quantification

In experiment 2, foliar ABA levels were measured three times during the various water treatments. Once initial rates of water potential were recorded from the single detached leaf, the leaf was covered with a plastic bag located inside paper bag, kept cool and brought to the laboratory within three hours. 0.02g of leaf tissue was excised and placed in 80% methanol at 20^{o}C overnight. The tissue was then ground using a hand-held electric blender for approximately three minutes. Adding 15µl of internal standard was conducted as explained by McAdam et al. (2012) "For all samples, the ratio of endogenous ion intensity to internal standard ion intensity was calculated. The product of this ratio and the amount of internal standard added was divided by the fresh weight of the tissue sample and adjusted for aliquot volume to determine the level of ABA per gram fresh weight in the leaf". After that, the residue was separated from the liquid and dried. 500 µl of 0.4% acetic acid was then added, followed by 300 µl of diethyl ether and the solution shaken twice. The top layer of liquid was decanted and dried using nitrogen gas. 150 µl of 5% MeOH in 1% acetic acid was applied. After that, samples were centrifuged at 13,000 rpm for 3 min. Finally, 50 µl of the top layer of supernatant was taken for combined ultra-performance liquid chromatography (UPLC.) and multiple-monitoring tandem analysis using a Waters Acquity H-series UPLC coupled to a Waters Xevo triple quadrupole mass spectrometer. A Waters Aquity UPLC BEH C 18

column (2.1 mm × 100 mm × 1.7 µm particles) was used to separate ABA, as previously described (McAdam et al. 2012, 2014).

4.2.5.5. Bulk density of soil core

After the above ground biomass of experiment 3 was harvested, soil cores for four blocks were collected using soil density rings (2.5 × 6 cm), and the soil porosity of the two soils assessed (Grable et al. 1968; Stirzaker et al. 1996). The two soils were that filtered with a 45µm sieve and not filtered, as above.

4.2.6. Statistical analysis

All data were statistically analysed using the SAS statistical analysis system (version 9.2; SAS Institute, Cary, NC, USA) software. Differences among means were tested using a revised L.S.D. test at the 0.05 level, after testing for homogeneity of variance and normality. The effect of inoculum treatment and time on growth measurements (height, number of leaves and stem diameter) was assessed using repeated measures ANOVA. The effects of inoculum and water treatments on physiological responses (photosynthetic, stomata conductance and water potential) were also explored via repeated measures ANOVA.

The effect of treatments on colonisation by *R. irregularis* and biomass were analysed using two way ANOVA respectively.

4.3. Results

4.3.1. Colonisation by AMF (*Rhizophagous irregularis*) and Local species and soil density

After the cherry rootstock samples in experiment 2 & 3 were harvested (week 31 and 29 respectively), the mycorrhizal colonisation levels were assessed.

In experiment 2, the absence of colonisation in non-inoculated cherry rootstocks was confirmed. In inoculated cherry roots, all mycorrhizal structures (hyphae, arbuscular and vesicles) were present under all water treatment conditions. The abundance of these structures was not significantly different between plants which received different water treatments for experiment 2 (Table 4.1a).

In experiment 3 cherry roots grown in both filtered and unfiltered soil showed evidence of AMF colonisation, but the roots in filtered soil had significantly higher percentage of colonisation than unfiltered soil (Table 4.1b). The bulk density of soil cores was significantly higher ($P = 0.04$) in the unfiltered compared to filtered soil (Figure 4.1).

Table 4.1: The mean (±SE) of percentage mycorrhizal colonisation of cherry rootstocks under different water treatments; (a) for experiment 2 (one way ANOVA, n=32) and (b)for experiment 3 (two way ANOVA, n=20).

(a)

	Inoculated cherry rootstocks (%)		
Colonisation structures	AW+EW	DW+EW	P value
Hyphae	61.33 ± 3.33	59.25 ± 1.73	0.5880
vesicles	36.42 ± 3.22	36.33 ± 1.76	0.9822
Arbuscular	29.92 ± 1.75	27.92 ± 3.69	0.6318

(b)

	Filtered soil (%)		Unfiltered soil(%)			
Colonisation structures	AW+EW	DW+AW	AW+EW	DW+EW	P value (soil inoculation)	P value (water treatments)
Hyphae	50.53 ± 5.03	44.93 ± 4.42	23.6 ± 1.13	36.8 ± 5.81	0.001	0.40
Vesicles	11.47 ± 1.90	12.93 ± 0.88	7.2 ± 2.34	2.67 ± 1.14	0.0005	0.37
Arbuscular	43.73 ± 6.48	35.2 ± 4.72	16 ± 1.41	27.87 ± 5.17	0.002	0.73

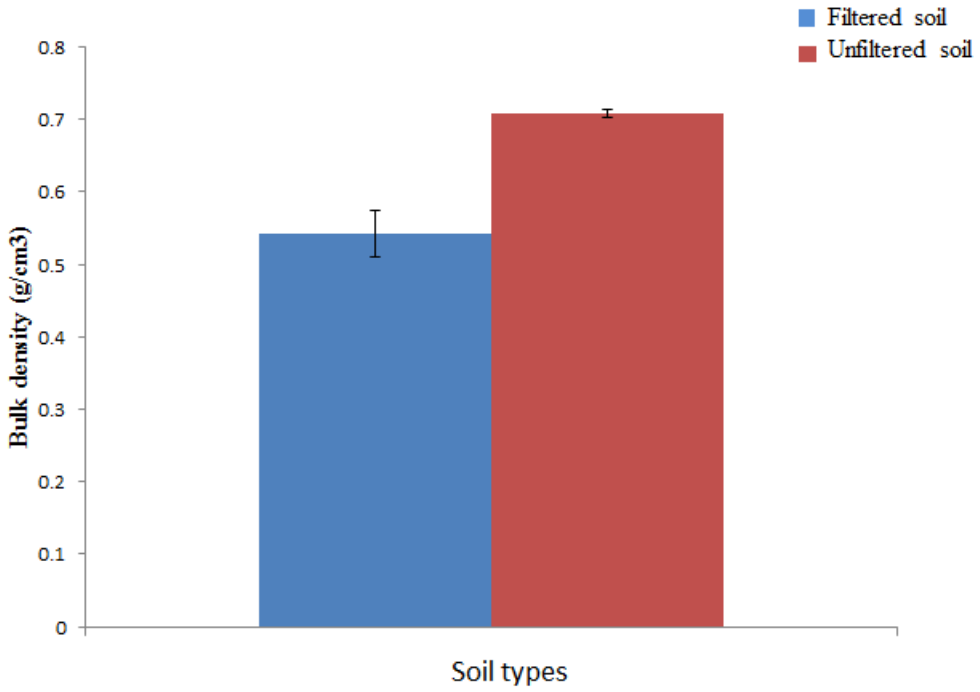

Figure 4.1: Mean value (± SE) of bulk density between filtered an unfiltered soil (n= 8) (one way ANOVA)

4.3.2. Survival

The survival rate of transplanted cherry rootstock cuttings in experiment 1 was significantly influenced by colonisation (P = 0.02). The survival rate for inoculated plants (assessed in week 9), was 65%, compared with 45% for non-inoculated plants.

4.3.3. Growth responses to colonisation

In general, plant roots grown in filtered soil, which had greater colonisation, led to the greatest plant growth in terms of height, stem diameter and production of significantly more leaves than those cultivated in unfiltered soil.

In experiment 1& 2, there were no significant effect of inoculum treatment on height growth (inoculum P = 0.45, inoculum' P = 84respectively).

In experiments 1 (data not displayed) and 2, there was no significant difference between leaf numbers for inoculated and non-inoculated plants (inoculum P =0.47, P = 0.07;

interaction 'time × inoculum' $P = 0.24$, $P = 29$, respectively) (Figure 4.2a). Neither were there any significant difference in numbers of leaves between inoculum treatments after the spread of the mite infestation disease in experiment 1 ($P = 0.18$).

However, in experiment 3, a significant difference on plant height growth was found to exist between filtered and unfiltered soil ($P = 0.0001$). In experiment 3, the plants grown in filtered soil which had greater colonisation led to production of significantly more leaves than those cultivated in unfiltered soil ($P = 0.001$). However, the interaction with time was not significantly different ($P = 0.14$) (Figure 4.2b).

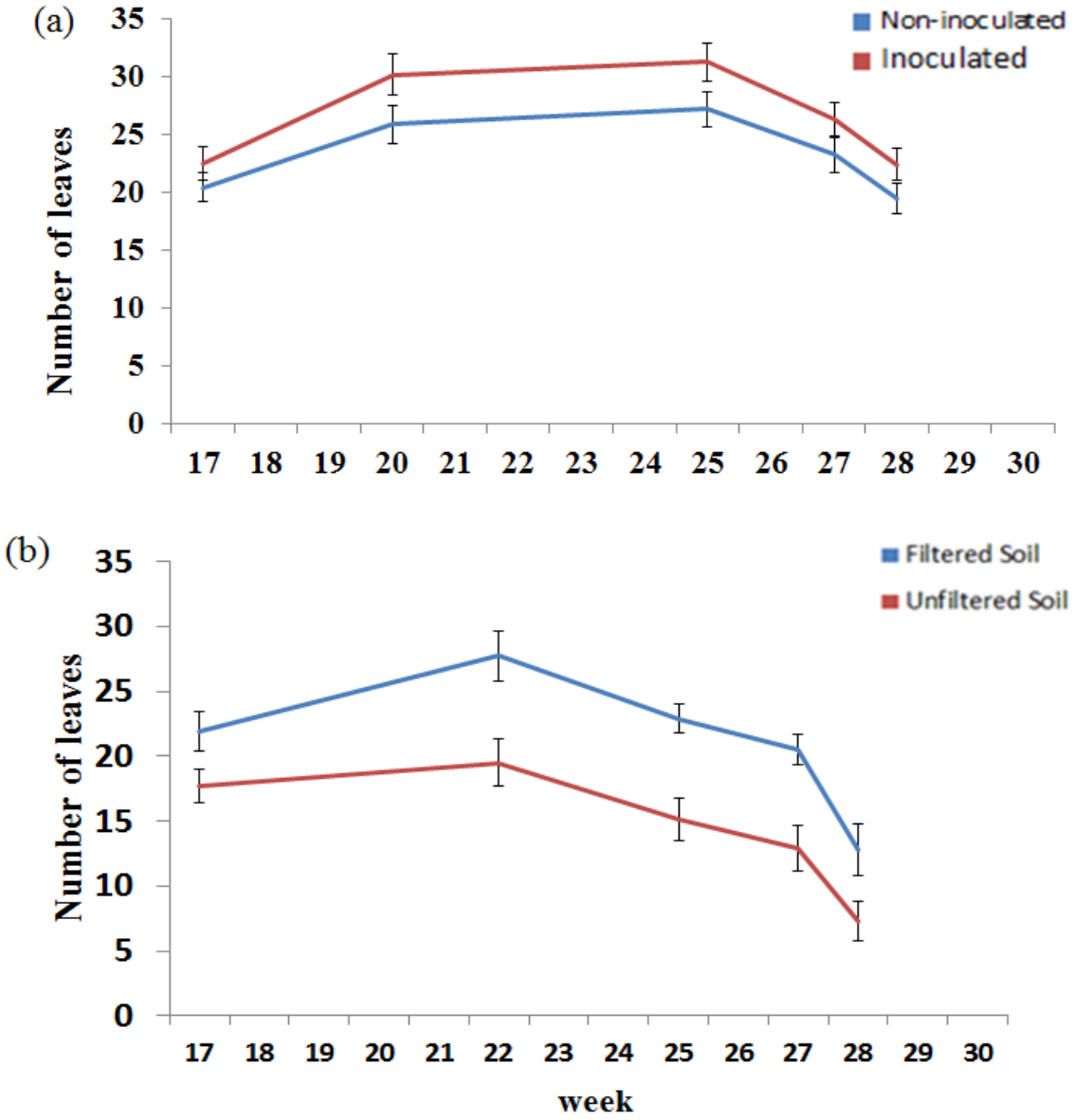

Figure 4.2: Mean value (± SE) of number of leaves, assessed at five time intervals; (a) cherry cuttings experiment 2 (n= 32); (b) cherry cuttings experiments 3 (n= 20).

Stem diameter (data not displayed), in experiment 1 showed no significant difference between inoculum and interaction with time (P = 0.38, P = 0.26 respectively). Similarly, in experiment 2, stem diameter measurement showed no significant difference between inoculum and interaction with time (P = 0.37, P = 0.08 respectively).

In experiment 3, the differences for stem diameter between filtered and unfiltered soil was increased significant (P = 0.006) (Figure 4.3).

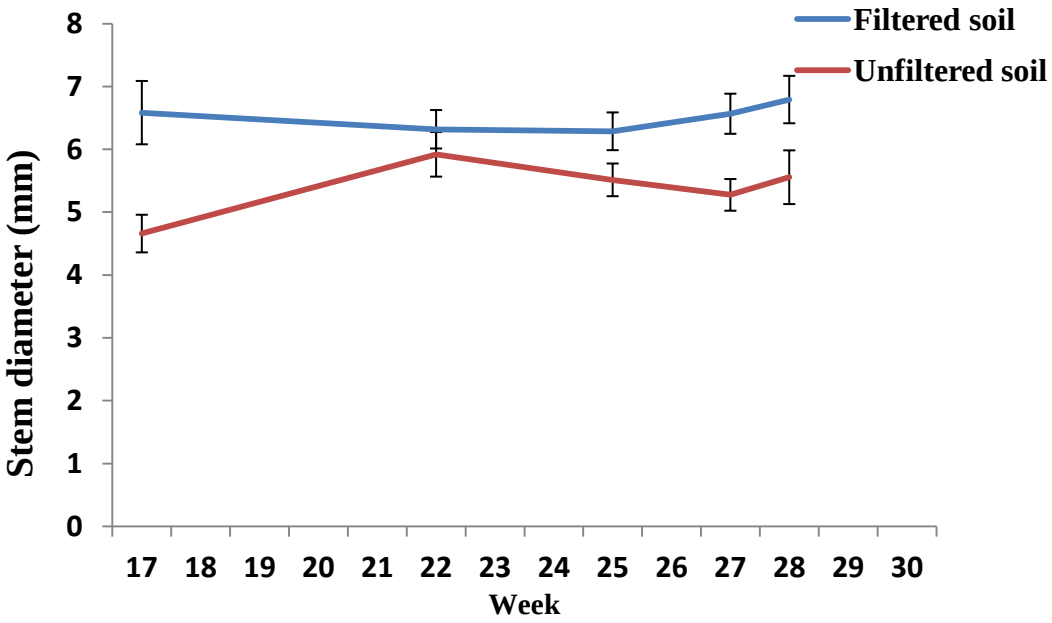

Figure 4.3: Mean value (± SE) of stem diamter (mm) in experiment 3.

4.3.4. Biomass

Biomass of plants in experiment 2 showed no significant difference with inoculation or water treatment for all three tissues (Table 4.2a). In experiment 3, plants grown in filtered soil had significantly higher biomass than those grown in unfiltered soil for all three tissues, but the water treatment did not significantly affect biomass (Table 4.2b).

Table 4.2: Mean (±SE) of biomass allocation to above-ground and root for both treatments, (Two way ANOVA) tables a & b for all experiments 2 (n= 32) & 3 (n=20)

(a)

	Non-inoculated (g)		Inoculated (g)		P value		
	AW+EW	DW+EW	AW+EW	DW+EW	Inoculum	Water treatment	Interaction between inoculum and water
Leaf biomass	1.80 ± 0.39	1.59 ± 0.14	2.23 ± 0.32	2.08 ± 0.28	0.16	0.56	0.91
Stem biomass	5.19 ± 0.70	4.49 ± 0.37	5.18 ± 0.28	5.06 ± 0.49	0.58	0.43	0.57
Root biomass	14.21 ± 2.88	13.10 ± 1.28	11.90 ± 1.15	8.86 ± 0.96	0.07	0.24	0.58

(b)

	Filtered soil (g)		Unfiltered soil (g)		P value		
	AW+EW	DW+EW	AW+EW	DW+EW	Inoculum	Water treatment	Interaction between inoculum and water
Leaf biomass	2.51 ± 0.60	1.56 ± 0.43	0.55 ± 0.45	0.99± 0.30	0.01	0.58	0.15
Stem biomass	7.13 ± 0.89	7.34 ± 0.50	3.69 ± 0.82	3.6 ± 0.48	0.001	0.93	0.82
Root biomass	27.69 ± 5.45	30.35 ± 2.22	6.89 ± 3.42	13.24 ± 2.82	<.0001	0.24	0.62

4.3.5. Physiological responses to water treatments

Photosynthetic rate 'net CO_2 uptake (A1500)' at ambient CO_2 concentration (400 µmol mol^{-1}) of cherry rootstock samples in experiment 2 was not significantly different due to inoculum

and water treatments and the interaction between treatments was also not significant (P = 0.09, P = 0.14, P = 0.10 respectively) (Figure 4.4a). In experiment 3, the photosynthetic rate of plants grown in filtered and unfiltered soil and different water treatments displayed no significant difference (P = 0.23, P = 0.49 respectively), but there was a significant interaction between inoculum and water treatments (P = 0.0003) (Figure 4.4b).

The rates of stomatal conductance in experiment 2 differed significantly depending on water treatment conditions *(*P = 0.01, Figure 4.5a). However, there were no significant differences between inoculum treatments, nor the interaction (inoculums with water treatments) (P = 0.56, P = 0.93 respectively). Stomatal conductance in inoculated plants that received AW recovered faster than in other plants under recovery 2 times (R2) (Figure 4.5a). The stomatal conductance of plants in experiment 3 showed no significant difference in filtered and unfiltered soil or for the water treatment (P = 0.23, P = 0.92 respectively). However, the interaction between type of soil colonisation and water treatment displayed a significant difference (P = 0.01) (Figure 4.5b).

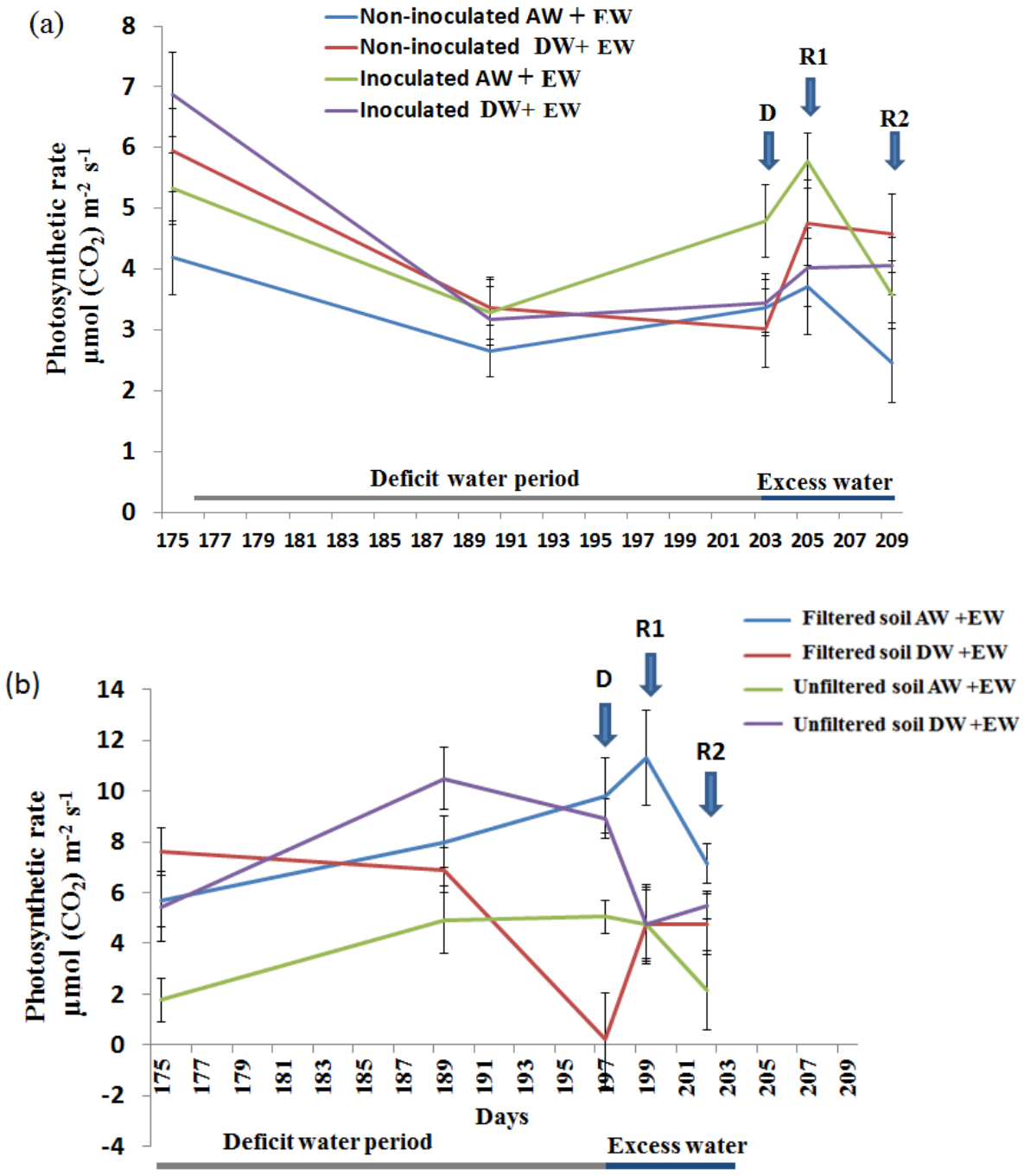

Figure 4.4: Mean value (± SE) of photosynthetic rate (A1500) (a) Experiment 2 (n=32), (b) Experiment 3 (n=20), (repeated measure ANOVA). D = data collected for deficit water period, R1 = data collected for recovery after supplying excess water for one day, R2 = data collected for recovery after supplying water for four days.

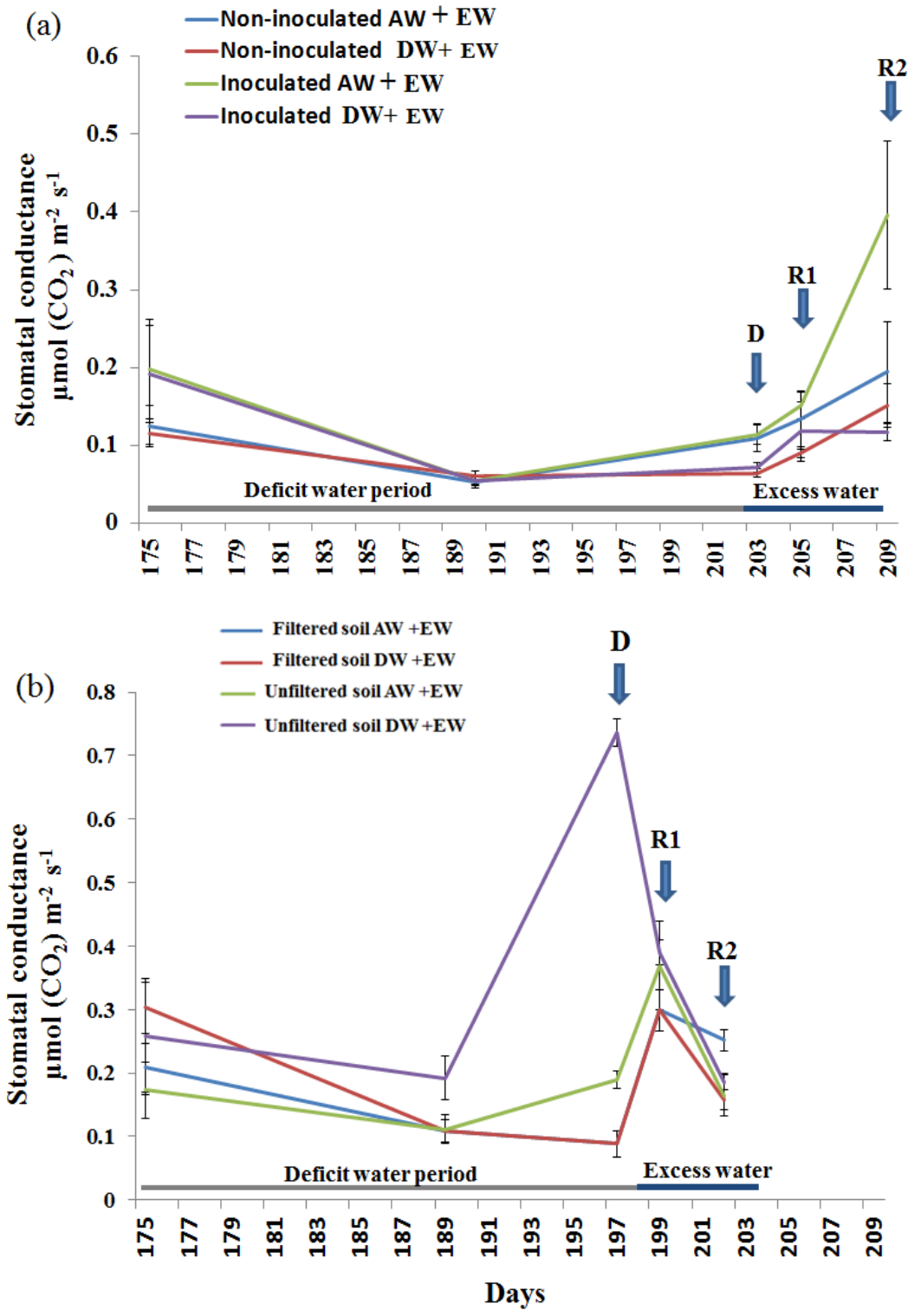

Figure 4.5: Mean value (± SE) of stomatal conductance (µmoll m^{-2} s^{-1}) (a) Experiment 2 (n= 32) and (b) Experiment 3 (n= 20) (repeated measurement ANOVA). D = data collected for deficit water period, R1 = data collected for recovery after supplying excess water for one day, R2 = data collected for recovery after supplying water for four days.

In experiment 2, water potential measurements indicated that there was a significant difference between water treatments. However, there was no significant difference between the inoculums or interaction ($P = 0.007$, $P = 0.88$, $P = 0.36$ respectively) (Figure 4.6a). Water potential of plants in experiment 3 was significantly affected by water treatments and interaction with soil type, but no difference was found between plants grown in filtered and unfiltered soil alone ($P = 0.001$, $P = 0.02$, $P = 0.26$ respectively) (Figure 4.6b).

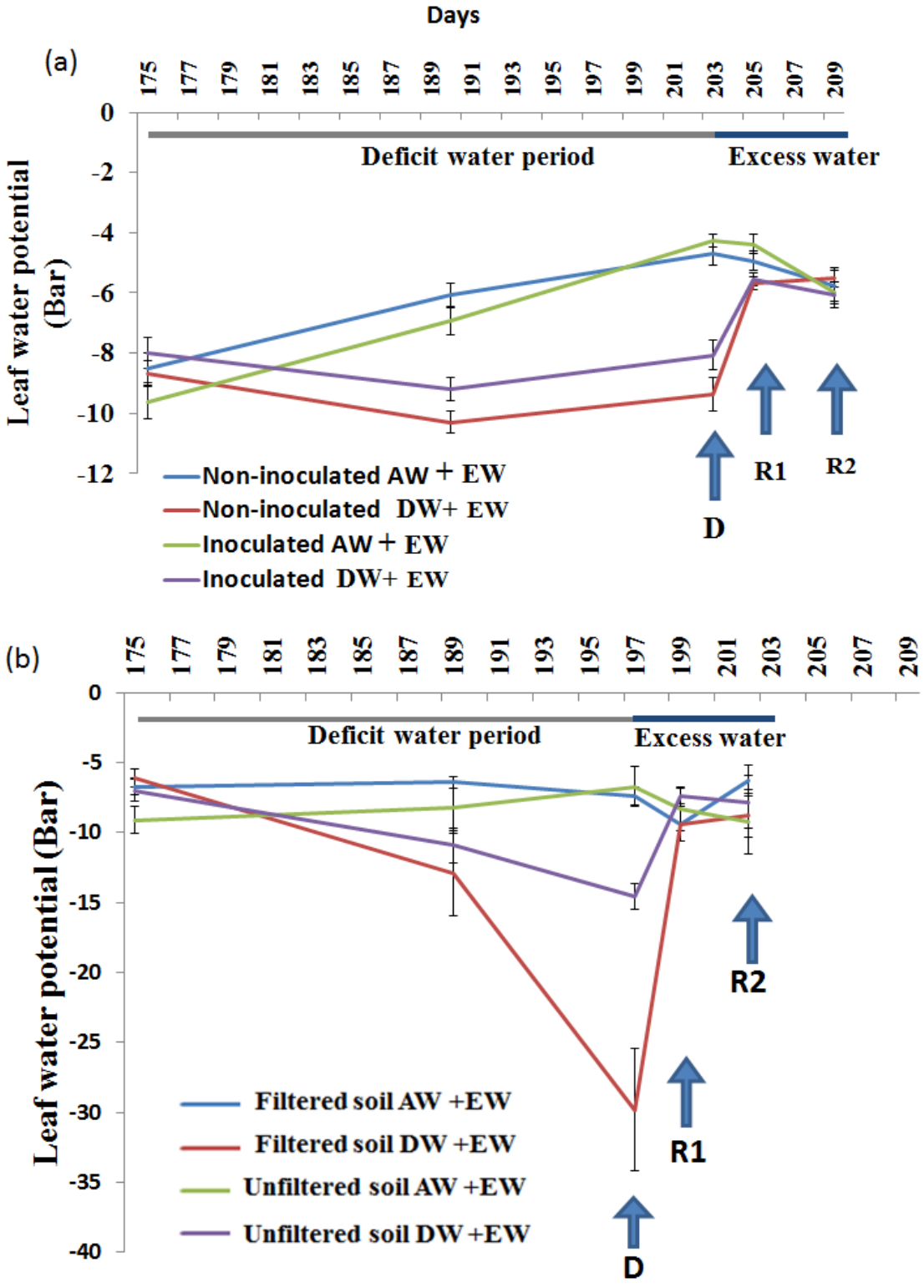

Figure 4.6: Mean value (± SE) of midday leaf water potential (Bar) (a) Experiment 2 (n= 32)and (b) Experiment 3 (n= 20) (repeated measurement ANOVA) D = data collected for deficit water period, R1 = data collected for recovery after supplying excess water for one day, R2 = data collected for recovery after supplying water for four days.

4.3.6. Abscisic Acid (ABA) content

The results show that there were no significant effect of inoculum, water treatments or their interactions on ABA (P = 0.13, P = 0.07 & P = 0.68 respectively) (Figure 4.7). Inoculated plants that had received adequate water were found to have a lower concentration of ABA on day 191. Although not significantly different, under drought condition (D) in day 203, there was a trend that ABA of inoculated plants (DW+ EW) was lower than non-inoculated, and plants which received adequate water (AW +EW) had lower ABA concentration, and both (AW + EW & DW) inoculated plants had low concentration of ABA during recovery 2 (day 209).

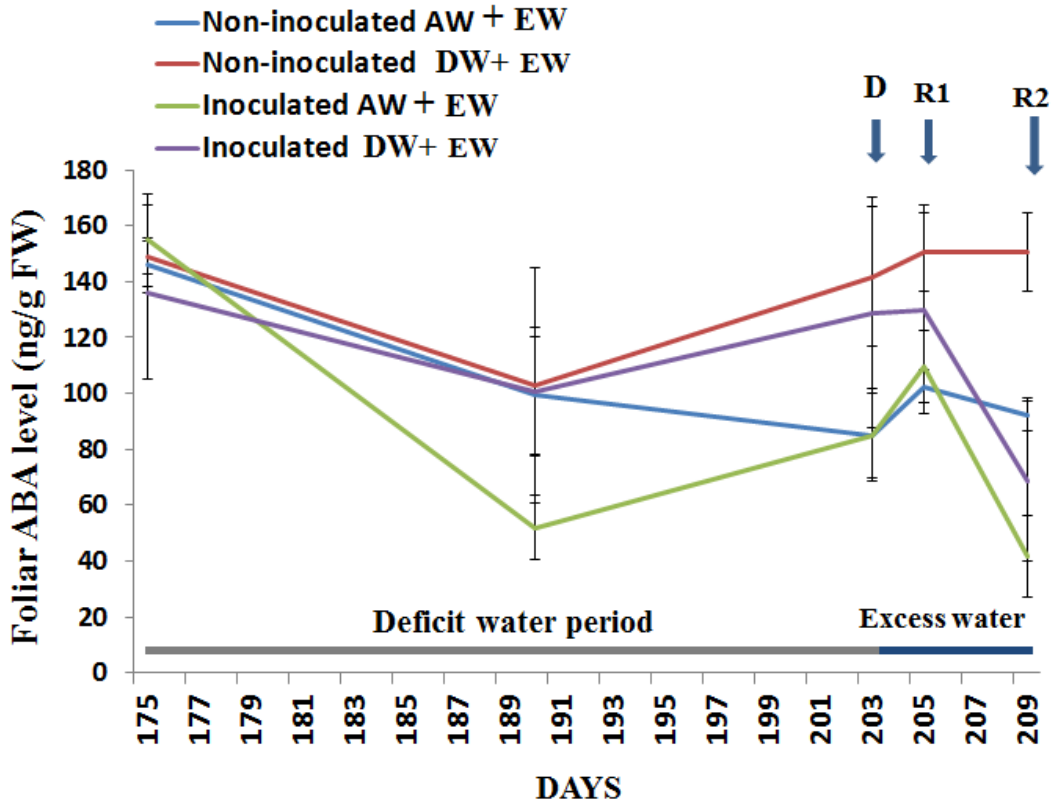

Figure 4.7: Mean value (± SE) of foliar ABA level (ng/g FW), collected four times under water treatment (drought stress from 175 to 191 to 302 day), excess water (recovery 1 from 203 to 205day), (recovery 2 from 205 to 209) (n=12) (repeated measurement ANOVA) D = data collected for drought period, R1 = data collected for recovery after supplying excess water for one day, R2 = data collected for recovery after supplying water for four days.

4.4. Discussion

4.4.1. *Rhizophagous irregularis* colonisation

The colonisation of plant roots by AMF in experiment 1 was not assessed due to the spider mite infestation. However, as the amount of spores that we inoculated was low, and the soil would have had high phosphate content in the form of Osmocote slow release fertilizer, we anticipate colonisation would have been low. This has led to poor colonisation in other studies (Akiyama et al. 2005; Schubert et al. 1986). Despite this, a treatment effect due to inoculation was detected, therefore there must have been a sufficient difference in colonisation rate to impact the survival as well (Estrada-Luna et al. 2000; Subramanian et al. 2006).

The high level of colonisation in experiment 2 was in line with expected levels, and was in agreement with previous studies (Calvet et al. 2004; Gao et al. 2001; Subramanian et al. 2006).

4.4.2. Local AMF species colonisation

However, in experiment 3, colonisation by local AMF species was detected but results were opposite to expectations. It was found that plants grown in filtered soil had a significantly higher rate of colonisation than those grown in unfiltered soil. Firstly, this reveals that filtering did not exclude a sufficient amount of inoculum to reduce colonisation, which could be due to small colonized fine root fragments passing through the 0.45 µm filter. Also, the soil bulk density showed significant differences: unfiltered soil had a significantly higher density than filtered. This means that unfiltered soil adversely affected the establishment of colonisation and plant growth. The result showed that increased soil bulk density was associated with decreased plants growth as reflected by the plant biomass (Table 4.2). These findings are consistent with those of several previous studies (Grable et al. 1968; Letey 1985; Stirzaker et al. 1996) that show bulk density and aggregate size had little effect on diffusion or concentration of O_2 and water desorption and thickness of water films limited root elongation. This may have been because of increased soil density. Filtered soil had less soil density, which leads to faster water drainage, so the plants which were under drought condition had more stress than those that were unfiltered as shown in leaf water potential

(Figure. 4.6b). This could be due to the water moisture drying faster (Garnier et al. 1998). Also in filtered soil it was found that the roots had more colonisation. This could lead to higher stress in host plants, as Rutto et al. (2002) stated that when inoculated peach seedlings were exposed to drought (water deficiency), there was a negative impact on survival, development and productivity.

4.4.3. Effect of AMF on establishment

The fact that in experiment 1 significantly more AMF inoculated plants survived than non- inoculated was consistent both with the hypothesis and previous findings (Gosling et al. 2006; Mukerji et al. 2000). This has positive implications for use of AMF inoculum commercially to increase success of cutting survival, even when regular fertilizer is used.

4.4.4. Effect of AMF colonisation on growth

This study hypothesised that inoculated cherry rootstock samples would exhibit faster growth, in terms of number of leaves, stem diameter and biomass, compared with non-inoculated plants. It was found that plants grown in filtered soil which had greater colonisation led to greater growth in terms of production of leaves, height and stem diameter and biomass. little information is known on potential roles of different AMF for growth and nutrient acquisition of sweet cherry, for example, (Bâ et al. 2000) stated that five tropical fruit trees (*Adansonia digitata, Aphania senegalensis, Anacardium occidentale, Balanites aegyptiaca, and Sclerocarya birrea*) acquired benefits from AMF (*Glomus aggregatum and Glomus intraradices*) inoculation. In addition, it has been found that in apple (*Malus domestica*), inoculated with *G. mosseae* singly and in combination with *G. macrocarpum* were more effective in increasing plant biomass than *G.macrocarpum* (Doud Miller et al. 1989). (Wu et al. 2011) found that in peach (*Prunus persica* L. Batsch) seedlings inoculated with *Glomus mosseae*, *G. versiforme*, and *Paraglomus occultum*, respectively, The beneficial effect role on nutrient uptake generally was greatest in the *G. mosseae* treatment, and suggesting that different AMF play different roles on plant growth, due to functional diversity of AMF. Unfiltered soil, which had less AMF colonisation, significantly affected growth and biomass and specifically the associated greater bulk density of unfiltered soil resulted in significantly less root biomass of the cherry rootstock samples (probably due to reduced root

length and limited root penetration). This is in keeping with the findings of Rai et al. (2010) that root growth increased in direct proportion to the decrease in bulk density of soils.

In experiment 2 the cherry rootstock samples were successfully colonized with *R. Irregularis* after transplant; however, growth and biomass were not significantly different. From this study it is clear that the function of *R. Irregularis* with sweet cherry rootstocks (*Prunus avium* L *x Prunus pseudocerasus*) is not effectively work. However, other studies suggested that AMF need to be colonized in the early stages of growth to receive the full benefits of the association with AMF (Calvet et al. 2004; Rutto et al. 2002).

4.4.5. Effect of AMF colonisation on physiological response

This study investigated physiological aspects related to water relations and drought tolerance in AMF and non-AMF plants subjected to adequate water and drought stress. A trend showed a slightly higher rates of photosynthesis in AMF plants under adequate water were detected in experiment 2. This increase in photosynthesis would be expected to result in higher concentration of carbohydrate contents in leaf symplasm which increases cytoplast osmolality in AMF plants (Augé 2001; Porcel et al. 2004). However, drought-exposed AMF plants showed a trend for lower photosynthesis than inoculated plants with adequate water, which could be as explained by Augé (2001) that AMF plants more successfully avoid drought stress with lower photosynthesis rates.

In experiment 3, the function of stomata conductance reduced in both filtered and unfiltered soil colonisation under different water conditions (AW, DW), day 197. As soil dries out plants must activate their defence system to be sure to absorb water as much as they can to enhance the host plant drought resistance (Song 2005). It has been reported that in various AMF plants (blue grama, cowpea, lettuce, rose, safflower, soybean and wheat) the rate of transpiration and stomata opening increased relative to non-inoculated plants (Augé 2001). In contrast, similar stomatal performance was shown for AMF and non-AMF plants in experiment 2. This was explained by Khalvati et al. (2005) who found the daily contribution of water to roots from the hyphal compartment was small (only approximately 0.15 ml plant $^{-1}$ day^{-1}) compared to transpiration of about 10 ml H_2O plant^{-1} day^{-1} which was averaged each day cycle. However, the role of AMF in water uptake ranges from plants that receive adequate water to those experiencing drought stress (Khalvati et al. 2005; Rutto et al. 2002).

According to Ruiz-Lozano et al. (1995), there are large differences in water uptake by AMF-plants that are due to differences in the extent of the hyphal network, and also differences in host plant response.

Due to the important role of ABA in stomata control, the measurements of this hormone were made following the changes in water treatment (before and after deficit and excess water treatments). ABA of AMF plants under DW-EW stress were showed a trend for lower (but not significantly) compared with non-inoculated plants exposed to the same stress, which agrees with previous studies for activating their defence system against drought stress (Duan et al. 1996; Estrada-Luna et al. 2000; Estrada-Luna et al. 2003). The observation that ABA of all plants decreased during the drought period when assessed at 191 days is difficult to explain; it would be expected that the ABA levels of the plants exposed to DW would increase. The change in watering regime from automated irrigation to hand-watering may have impacted physiological status. At the end of the DW period (203 days),. In the recovery period during EW exposure, the ABA levels of the AMF-inoculated plants decreased substantially between R1 and R2 assessments, which reflects a rapid recovery from drought. The fact that the non-inoculated DW-EW treatment plants retained a higher ABA level during this period reflects non-recovery, as also corresponds to the greatest reduction in water potential for plants of this treatment. This indicates that the presence of AMF may assist plants to recover from drought.

In experiment 2, AMF assisted host plants to maintain higher (less negative) leaf water potential. Plants that were exposed to DW had lower (more negative) water potential compared to the AW plants, however the water potential of inoculated DW-exposed plants was a trnd for less negative then for the non-inoculated DW-exposed plants. This was consistent with other studies that found AMF reduced drought stress in host plants by postponing declines in leaf water potential (Augé 2001; El-Tohamy et al. 1999; Porcel et al. 2004; Song 2005). This could have occurred due to one of these mechanisms, in that AMF helped host plants through drought avoidance tolerance by hyphal water uptake or it change the root morphology or soil structure this could occur AMF allow plants to remain more hydrated than non-inoculated (Augé 2001; Porcel et al. 2004). Interestingly, in experiment 3, under soil dries out and soil water potential becomes more negative filtered soil colonised plants that subjected to drought stress, day 197, showed a trend for higher leaf water

potential which led to reduce the performance of photosynthesis and stomata conductance for activating their defence system (Song 2005).

4.5. Conclusion

This study has investigated the effects of AMF colonisation on the survival and growth of cherry rootstock, as well as its ability to better regulate water balance under conditions of water stress. This has been done by comparing colonized plants and non-colonized ones. The effect of *R. irregularis* on growth was not significant, however it significantly increased the survival rates of cherry cuttings, and there was a trend for enhanced photosynthesis and stomatal conductance. In addition, filtered soil that had high level of colonization resulted in significantly more growth in terms of production of leaves, height and stem diameter, while also increasing the photosynthetic rate and stomatal conductance under different water treatments.

The main aim of this research was to investigate whether AMF colonisation of cherry rootstocks assisted water relations of plants under water stress. In summary, the hypotheses could be more thoroughly tested *by* future studies should seek to conduct similar experiments with higher levels of colonisation in order to address the hypotheses fully. It would be advisable in future studies to inoculate the cuttings during experiment establishment, and then add further inoculum during the transplantation phase.

Chapter 5: Effect of AMF on Water Relations of Tomato

5. 1 Introduction

The main objective of this study was to determine if the presence of mycorrhiza on tomato plants leads to better regulation of water balance under conditions of water stress than for plants without mycorrhizae. The widespread symbiotic relationship between arbuscular mycorrhizal fungi (AMF) and the roots of plants has long been associated with improved plant performance (Gosling et al. 2006). The fungus receives carbohydrates from the plant which, in turn, receives increased water uptake and nutrient absorption (Parniske 2008). The diameter of arbuscular mycorrhizal fungal hyphae is typically 2–5 μm, so it can penetrate soil pores inaccessible to root hairs (10–20 μm diameter), and thereby absorb water that is not available to non-AMF plants. Thus, in this association, the fungus supplements the role of the plant's root hairs and acts as an extension of the root system (Khalvati et al. 2005).

AMF can increase plant water and nutrient uptake in conditions of moderate drought stress (Augé 2001; Fulton 2011; Schreiner et al. 2007). Under conditions of both adequate water and drought, mycorrhizal plants generally show higher stomatal conductance and an increased rate of transpiration (Augé 2001; Fulton 2011). These fungi can effect an increase in tolerance of water stress by increasing root hydraulic conductivity: the fungi enable the roots to extract water from small pores in the soil and regulate both stomatal conductance and osmotic adjustment in the host plants (Fulton 2011). In addition, mycorrhiza affect the size of host plants, making them larger, with longer roots that help to access a greater quantity of soil water and so scavenge water more effectively (Augé 2004; Schreiner et al. 2007).

Herbaceous plants such as tomato are sensitive to drought at all developmental stages (Wahb-Allah et al. 2011). Additionally, excess water is not always beneficial. The tomato is a soft fruit which suffers from water-related disorders such as fruit cracking that are caused when there is rapid water uptake into the fruit at the same time as ripening or other factors reduce the strength and elasticity of the tomato skin (Peet 2008).

Subramanian et al. (2006) found that tomato plants *Lycopersicon esculentum* L. (Variety PKM-1) inoculated by *Glomus intraradices*, today known as *Rhizophagous irregularis*

(Formey et al. 2012; Sashidhar et al. 2012), and then subjected to different levels of drought conditions in the field, had greater growth (height and number of branches) than non-inoculated plants. When the inoculated tomato plants were exposed to severe drought stress, they showed more intensive colonisation and a significant increase in the biomass of their roots and shoots under all levels of drought conditions. Furthermore, foliar P was enhanced by up to 30–40% more than in the non-mycorrhizal tomato plants (Kaya et al. 2003; Sheng et al. 2008; Subramanian et al. 2006). Mycorrhizal dependency 'is the degree of plant growth change associated with arbuscular mycorrhizal colonisation' (Tawaraya 2003). This is more pronounced under drought conditions as the colonisation enhances the leaf relative water content (RWC). This colonisation also results in improved plant N and P nutritional status which in turn increased production of tomato fruit by 24% under severe drought conditions, 23.1%, under moderate conditions and 12.1% when drought conditions were mild (Asrar et al. 2012; Foo et al. 2013; Subramanian et al. 2006). In addition, it was reported that mycorrhiza enhanced fruit quality by decreasing the acidity and increasing ascorbic acid content (Subramanian et al. 2006).

While much is known about the response of AMF-plants to drought, the capabilities of mycorrhizal plants under conditions of short-term excess water is unknown, therefore a study was designed to both confirm the beneficial effect of AMF on water stressed plants, and investigate the impact of AMF on plants receiving excess water. This was investigated in conjunction with low water stress to examine the ability of plants to tolerate the transition from low to high water, as a rapid transition can lead to splitting in some soft fruit (Measham et al. 2012).

We investigated the impact of mycorrhizal presence and soil type on a range of variables (growth, biomass production, photosynthetic rate, water potential and stomatal conductance) when plants experienced deficit or excess water availability. It was hypothesised that under deficient or excess water conditions mycorrhizal plants would have considerably higher water absorption potential, photosynthetic rate, stomatal conductance and gas exchange than their non-mycorrhizal counterparts. Additionally, it would be expected that tomato plants colonized by *R. irregularis* would have faster growth in terms of number of leaves, stem diameter and biomass, compared with non-mycorrhizal plants. While these studies were conducted prior to fruit maturity, it would be expected that enhanced photosynthesis and

stomatal conductance during the low water treatments may reduce the plant shock when later exposed to excess water condition which may protect fruit from splitting later in plant development.

5.2 Material and methods

Three separate experiments were designed to assess the objectives. All experiments were conducted in glasshouses at the University of Tasmania Sandy Bay campus, Southern Tasmania in 2013. The first experiment was conducted in May to October 2013, while the second and third experiments were conducted from September to December 2013. The difference between the second and third experiment was primarily the potting substrate (experiment two substrate included peat and perlite while the third did not).

5.2.1 Plant material

Wild-type tomatoes (*Lycopersicum esculentum* cv 76R) grown from seed were used in all experiments. Seeds were prepared and sterilized by soaking in 4% sodium hydrochloride for 15 min. They were then rinsed twice in distilled water and set on moist filter paper in a room at 25^0C to germinate for 5 – 7 days (Gao et al. 2001).
Once germination had occurred, plastic plant pots of approx. 10 cm diameter were filled with potting medium. The media was prepared as follows for each experiment;

- Experiment 1& 3: the medium was prepared by filling the base with a 3 cm layer of gravel and then a 6 cm layer of vermiculite.
- Experiment 2: plastic pots were filled with a 3 cm layer of gravel and then a 6 cm layer of potting mix, consisting of equal parts of sterilized (autoclaved for 1 hr. at 121°C) vermiculite, perlite and sphagnum peat (1:1:1, v/v/v).

5.2.2. Growing conditions

During the experimental periods, all plants were maintained in a glasshouse where the temperature was controlled by a reverse-cycle air conditioner (23/18^o C day/night) (Model #FTXS60F, Daikin). Both mycorrhizal and non-mycorrhizal pots in all experiments were fertilized with Hoagland's solution (Omar 1997), 25 ml three times per week for the first 5 weeks. Then the remaining weeks the plants received 35 ml per pot three times a week.

For the first 13 weeks of experiment 1, the plants were fertilized with regular Hoagland solution, but with one-quarter strength phosphorus. After the establishment of AMF colonisation was checked in week 13 (and found to be low), during the remaining six weeks the plants received a modified Hoagland's solution with very low phosphorus content (0.05 µM NaH2PO4) (Foo et al. 2013). For experiments 2 and 3 plants received the very low phosphorus solution for the entire duration.

Due to the development of early blight disease (caused by fungus *Alternaria solani)* (Chaerani et al. 2006; Fritz et al. 2006) from week 17 in experiment 1, copper liquid (5 ml L^{-1}) was applied several times to the foliage of all plants as a fungicide spray. For plant health, copper liquid (5 ml L-1) was also applied several times to the foliage of all plants in experiments 2 and 3 for the duration of the experimental period. Plants that were severely infected were removed from the experiment and the number of blocks reduced, as outlined below.

5.2.3 Experiment design

All experiments were conducted as randomized complete blocks in a 2 x 2 factorial design. Experiment 1 initially had 12 replicate blocks (blocked by bench location) making a total of 48 individual plants in all. The number of replicate blocks was reduced to five following development of early blight disease which affected some of the plants (28 plants). Experiments 2 and 3 had 5 replicate blocks, making a total of 20 plants in each experiment.
Treatments included two water supply levels: adequate water followed by excess water (AW+EW), and deficit water followed by excess water (DW+EW) and two inoculum levels (with and without inoculation) which will be further outlined below.

5.2.4 Treatments

5.2.4.1. Fungal material and inoculation

All plants selected for inoculation were inoculated with the spores of *R. irregularis* (Premier Tech Pty. Ltd, Quebec, Canada; 500 spores per g in a sand matrix). This occurred by mixing about 400 g of inoculated root fragments of spring onion (*Allium fistulosum*) plants which had been inoculated several weeks earlier with the spores. Moreover, a solution of 0.1 g/ml of

inoculum (produced by diluting 50g of *R. irregularis* product in 500 ml of water) was added to each inoculated pot as a 10 ml dose at week 2, 4, 6 and 8 (Foo et al. 2013; Gao et al. 2001). In addition, in experiment 1, additional fungal spores were added by drilling a hole in each inoculated pot then adding around 6 g of the spore matrix in week 14.

All non-inoculated tomato seedlings received a control solution (a custom-ordered product from Premier Tech Pty. Ltd. which included the sand only, not spores). In addition, non-inoculated plants in experiment 2 & 3 received root fragments of spring onion (*A. fistulosum*) planted without inoculation, which were added in the same manner as the treated plants.

5.2.4.2. Water

All plants were initially watered adequately to avoid water stress until growth was well established. Irrigation levels were determined from the water holding capacity was determined as for chapter 4.

Deficit water (DW) include an initial period of low water (50% water holding capacity), followed by excess water (EW) (150% water holding capacity). Low water treatments were applied in week 19, 9 and 13 for experiments 1, 2 and 3 respectively which was imposed for duration of 9, 10, and 9 days for experiments 1, 2 and 3 respectively. DW was considered sufficient from the difference in leaf water potential between drought-exposed and non-drought-exposed plants. EW was maintained for 4 days.

5.2.5 Assessments

5.2.5.1. Root colonisation

Root colonisation by AMF was estimated at the conclusion of each experiment by using the ink and vinegar method (Vierheilig et al. 1998), as described in chapter 4.

5.2.5.2. Growth responses

During all experiments the growth of plants was assessed regularly by determining plant height using a meter stick, and stem diameter 3 cm above ground of tomato seedlings shoots

with digital calipers. The number of fully expanded leaves on each of the tomato seedlings was also recorded. At the conclusion of the experiment, biomass of dry leaves, stems, and roots was measured after tissues were dried in the oven for five days at 40 ^{0}C.

5.2.5.3. Physiological responses

Gas exchange from fully expanded leaves and leaf water pressure potential (LWP) were measured between 10 am and 2 pm. These assessments were repeated three times during each experiment at the following time points: "pre-treatment" (time 0), "mid-treatment" (at the completion of low water treatment, which was day 139, 73 and 91 for experiment 1, 2 and 3 respectively), and "end-treatment" which was after recovery from the 4 day excess water treatment. Gas exchange and leaf water potential were measured as described in chapter 4.

5.2.6. Statistical analysis

All data sets were statistically analysed using SAS statistical analysis system (version 9.2; SAS Institute, Cary, NC, USA) software. Differences among means were tested using a revised L.S.D. test at the 0.05 level.

The effect of inoculum treatment and interaction with time on growth measurements (height, number of leaves and stem diameter) were determined using ANOVA repeated measures analysis. In addition, the effects of inoculum and water treatments (and interaction between them) on physiological responses (photosynthetic, stomata conductance and water potential) were also explored via ANOVA repeated measures. Treatment effects on colonisation by *R. irregularis* and biomass were analysed by ANOVA (one way and factorial respectively).

5.3 Results

5.3.1 Colonisation by AMF (*R. irregularis*)

After the tomato seedlings were harvested, mycorrhizal colonisation was assessed and the complete absence of colonisation in non-inoculated tomatoes was confirmed. In inoculated tomatoes, all mycorrhizal structures (hyphae, arbuscular and vesicles) were present under both water treatments conditions. Hyphae were most abundant, followed by arbuscules and vesicles in both water treatments (Figure 5.1), and there were no significant difference

between mean abundance of these structures for plants allocated to different water treatments (Table 5.1).

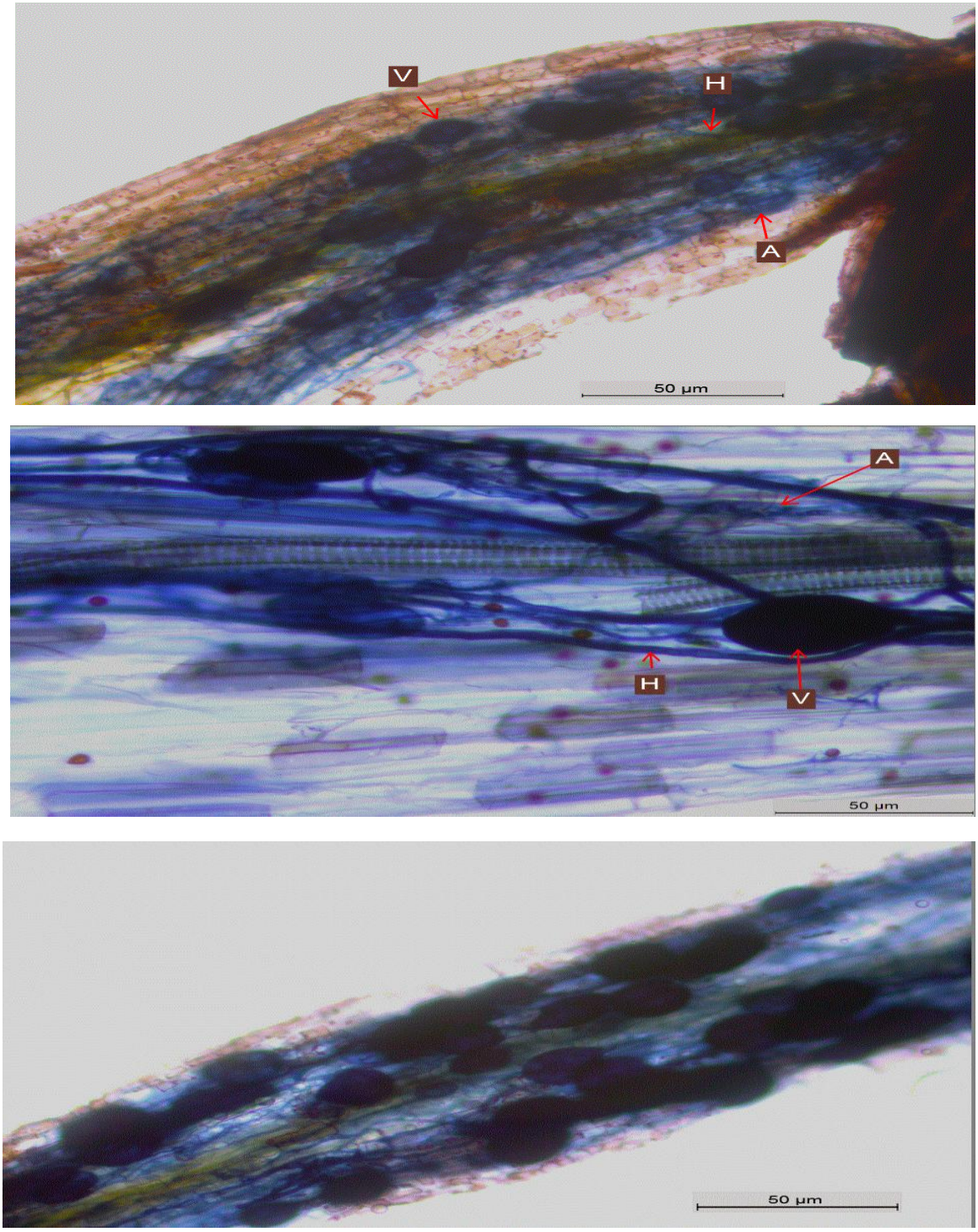

Figure 5.1: Micrograph of tomato roots colonized by *R. irregularis*, stained with ink and vinegar. Arrows indicate (H) Hyphae, (A) Arbuscules & (V) Vesicles (×200 magnification) (scale bar represents 50 µm).

Table 5.1: The mean (±SE) percentage mycorrhizal colonisation of plant roots under both water treatments (One way ANOVA) n= 20 (a, b, c for experiment 1, 2, 3).

(a)

	Inoculated Tomato Seedlings Roots Experiment 1		
Colonisation Structures	AW+EW	DW+EW	P values
Hyphae	74.23 ± 8.07	74.73 ± 5.56	0.96
Vesicles	45.93 ± 8.49	43.07 ± 8.89	0.82
Arbusculer	58.20 ± 8.03	60.80 ± 8.18	0.83

(b)

	Inoculated Tomato Seedlings Roots Experiment 2		
Colonisation Structures	AW+EW	DW+EW	P values
Hyphae	61.33± 3.51	56.93± 3.08	0.19
Vesicles	39.33± 4.69	28.00 ± 3.43	0.11
Arbusculer	55.20± 4.31	48.40 ± 3.64	0.24

(c)

	Inoculated Tomato Seedlings Roots Experiment 3		
Colonisation Structures	AW+EW	DW+EW	P values
Hyphae	80.40± 1.94	85.47± 1.94	0.39
Vesicles	36.13± 6.45	44.27± 1.69	0.26
Arbusculer	68.40± 4.43	72.00± 2.56	0.50

5.3.2 Growth responses to colonisation

Height growth of plants from all three experiments was effected by time significantly ($P < 0.0001$), which was expected. While the average height of inoculated tomato plants in experiment 1 was consistently higher than non-inoculated plants, it was not significantly different (Figure 5.2a). However, in experiment 2 and 3, inoculated plants were significantly taller than non-inoculated plants (Figure 5.2 b & c).

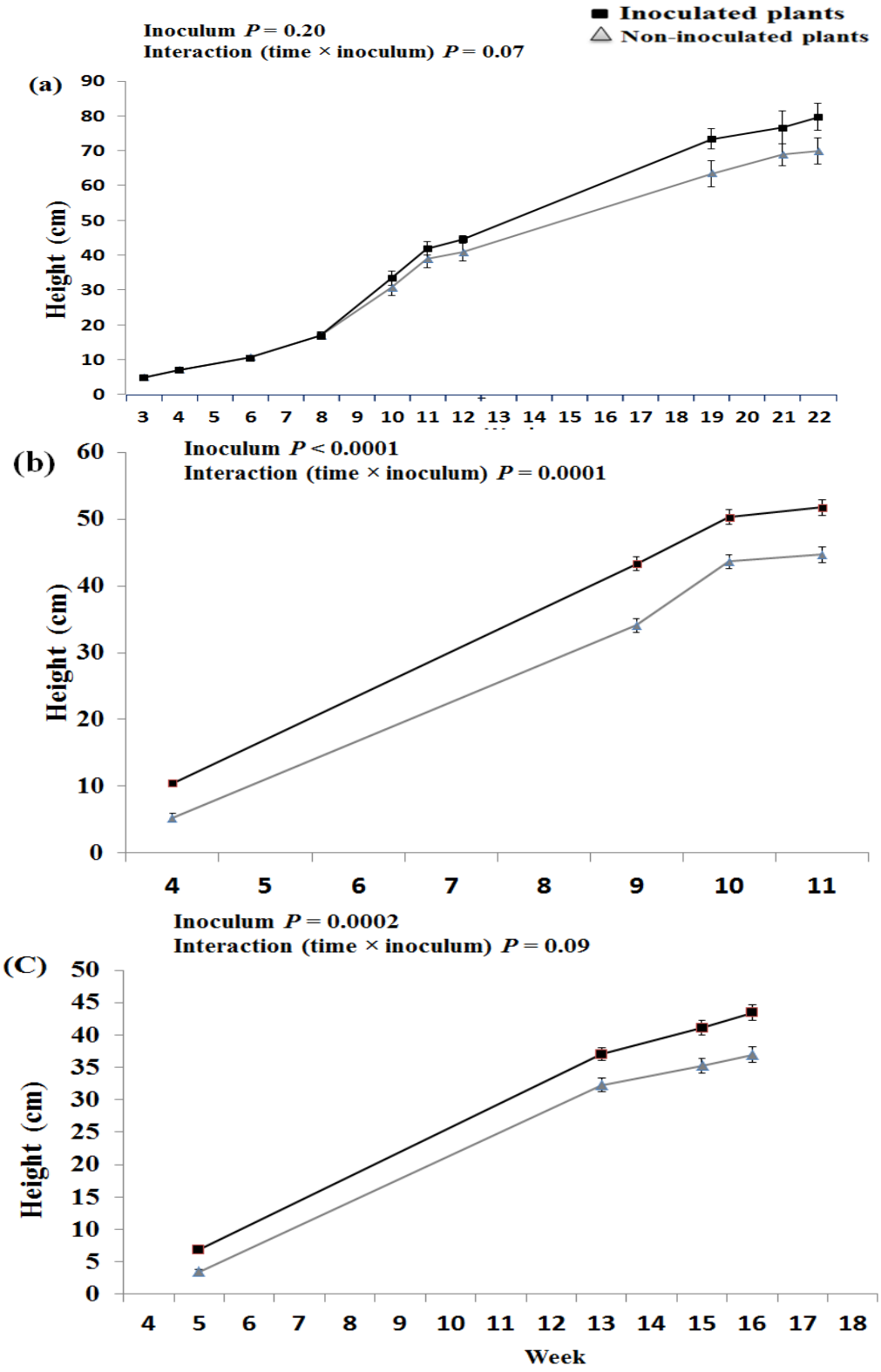

Figure 5.2: Represents the mean (±SE) of height (cm) of shoot above ground in three experiments, which were assessed at ten different times during experiment 1 (n=20) (a), and in (b) & (c) assessed four times in week (4, 9, 10, and 11) & (5, 13, 15 & 16) for experiments (2 & 3 respectively) (n=20). Data analysis was conducted with repeated measurement ANOVA.

The number of leaves also increased significantly with time in all three experiments. In experiment 1 (data not displayed) the difference between leaf number was not significant between inoculated and non-inoculated plants (inoculum $P = 0.79$, interaction 'time × water treatment' $P = 0.81$). However, in experiment 2 & 3 inoculated plants had significantly more leaves (Figure 5.3a & b).

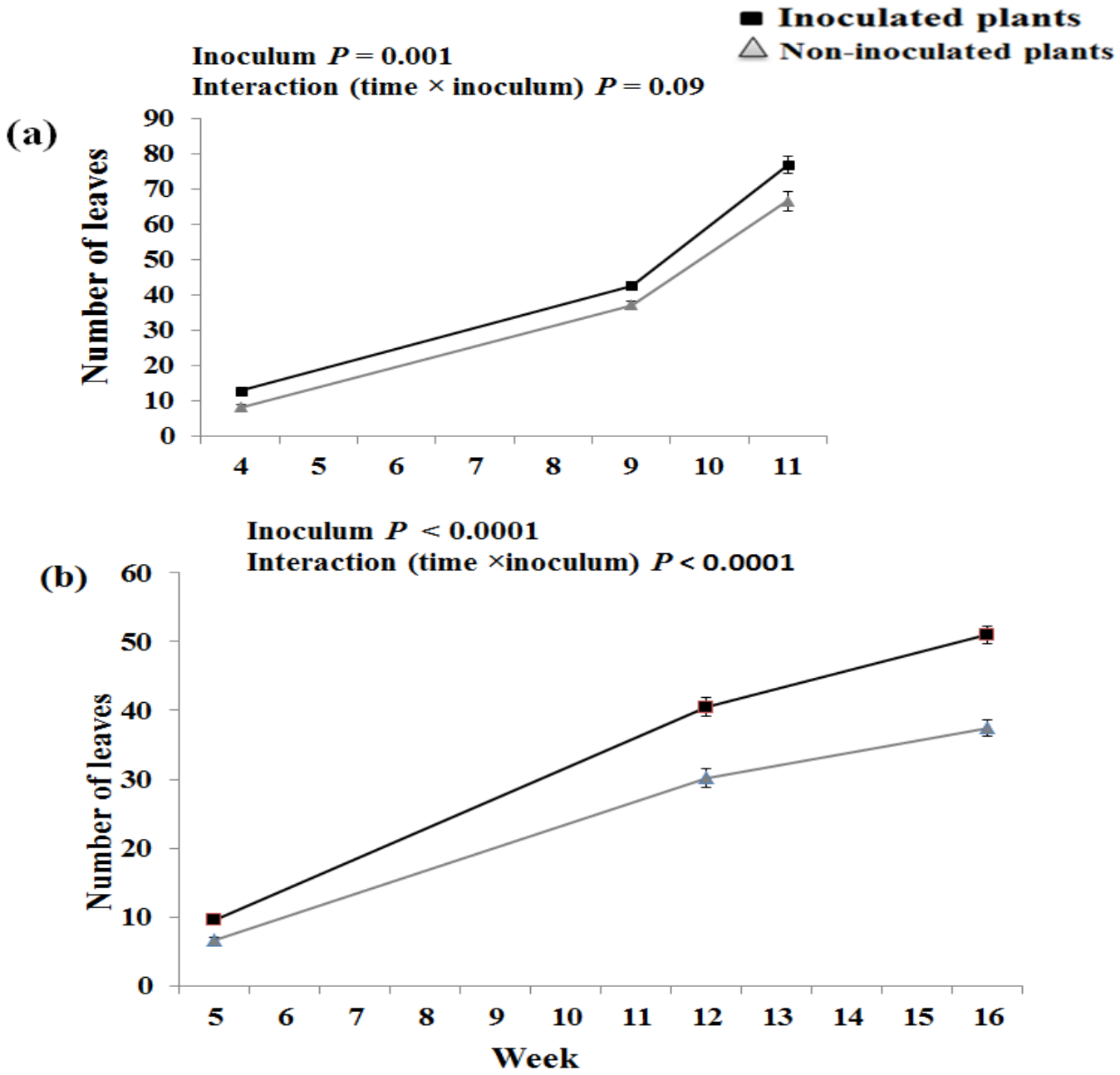

Figure 5.3: Represents the mean (±SE) of number of leaves (a) Experiment 2. (b) Experiment 3. *Three different weeks per experiment (n=20) (repeated measurement ANOVA)*

In experiments 1 & 2, the stem diameter was not significantly affected by inoculum treatment (inoculum P = 0.53 & interaction with time P = 0.32, inoculum P = 0.06 & interaction with time P = 0.28 respectively) (Figure 5.4a &b). However, in experiment 3, inoculated plants had significantly larger stem diameter compared with non-inoculated plants (P = 0.0009) and the interaction with time was not significant (P = 0.43) (Figure 5.4c).

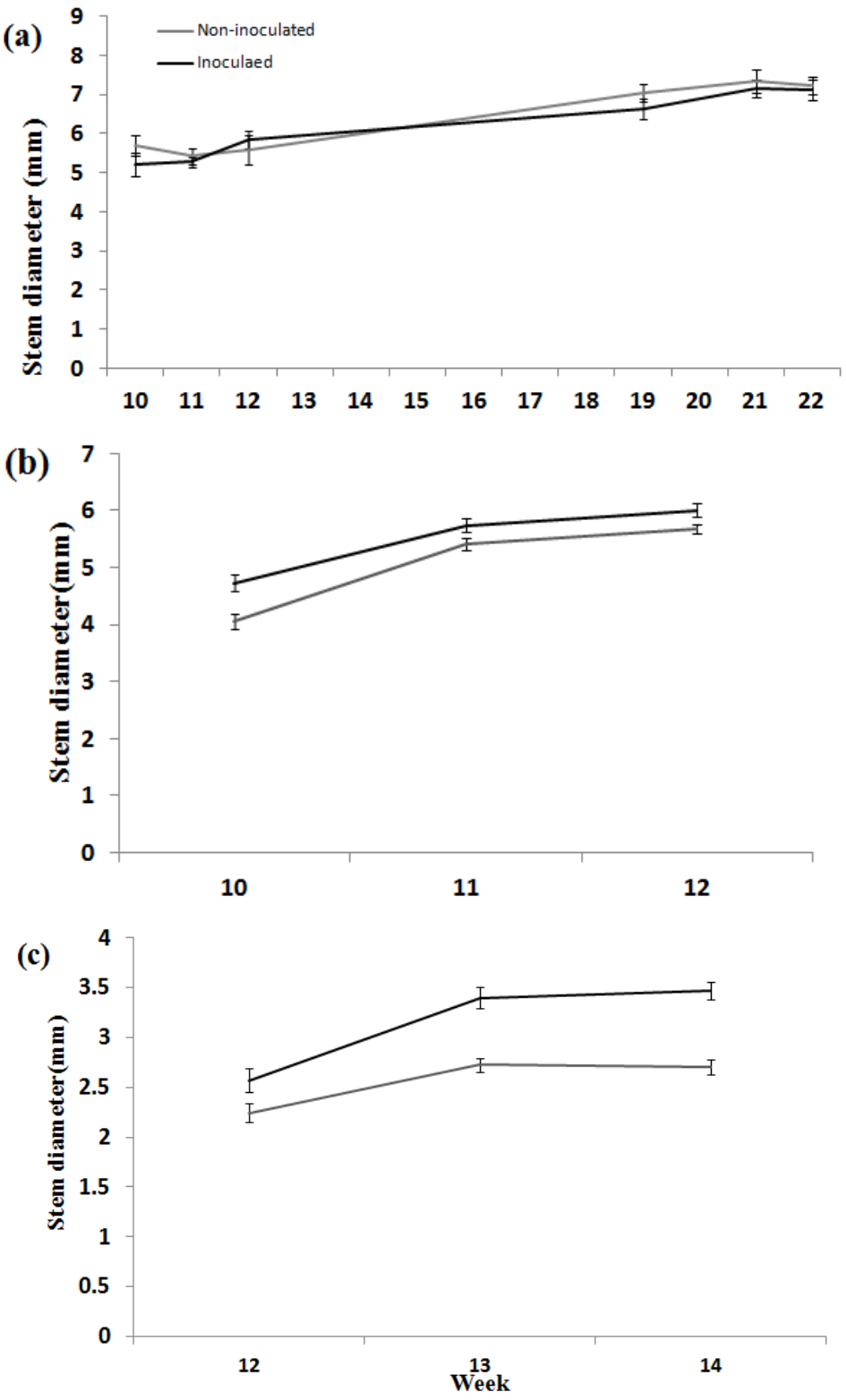

Figure 5.4: Represents the mean (±SE) of stem diameter (mm) (a) Experiment 1 (n=20). (b) & (c) Experiment 2 & Experiment 3 respectively (n=20) (repeated measurement ANOVA)

5.3.3 Biomass

Leaf, stem and root biomass of inoculated tomato plants compared with non-inoculated in experiment 1 had no significant differences due to the inoculum and water treatments (Table 5.2a). However, in experiment 2 inoculated plants had significantly higher leaf and stem biomass, while root inoculation treatment had no significant affection on root biomass (Table 5.2b), and all three tissue were significantly higher for inoculated plants in experiment 3 (Table 5.2c). The water stress treatment significantly reduced leaf and stem biomass in experiment 2 (Table 5.2b). There was no significant interaction between water treatment and inoculum on biomass of any tissue for any of the three experiments.

Table 5.2: Mean (±SE) biomass (g) allocation to above-ground and root for both treatments, (Two way ANOVA) tables a, b, c n = 20 for all experiments 1, 2, 3

(a)

	Non-inoculated		**Inoculated**		**P values**		
	AW+EW	**DW+EW**	**AW+EW**	**DW+AW**	**Inoculation**	**Water treatment**	**Interaction between inoculum and water**
Leaf biomass	5.76 ± 0.18	4.65± 0.059	5.87 ± 0.68	5.62 ± 0.55	0.26	0.16	0.37
Stem biomass	8.46 ± 0.72	6.32± 0.48	8.98 ± 0.82	8.71 ± 0.97	0.08	0.14	0.24
Root biomass	21.35 ± 1.76	17.77± 0.36	22.00 ± 3.50	23.35±2.21	0.21	0.62	0.32

(b)

	Non-inoculated		**Inoculated**		**P values**		
	AW+EW	**DW+EW**	**AW+EW**	**DW+EW**	**Inoculation**	**Water treatment**	**Interaction between inoculum and water**
Leaf biomass	3.97 ± 0.23	3.37 ±0.12	4.43 ± 0.10	3.78±0.14	0.01	0.001	0.87
Stem biomass	4.55 ±0.19	3.96 ±0.18	5.94 ±0.23	4.77 ±0.14	0.0008	0.005	0.29
Root biomass	7.55 ±0.76	6.90 ±0.58	9.31 ±0.51	8.74 ±0.69	0.07	0.51	0.96

(c)

	Non-inoculated		Inoculated		P values		
	AW+EW	**DW+EW**	**AW+EW**	**DE+EW**	**Inoculation**	**Water treatment**	**Interaction between inoculum and water**
Leaf biomass	0.49 ± 0.10	0.37 ±0.07	0.88 ± 0.12	0.89±0.08	0.0002	0.55	0.51
Stem biomass	0.85 ±0.12	0.69 ±0.05	1.33 ±0.18	1.29 ±0.11	0.0006	0.45	0.68
Root biomass	0.65 ±0.13	0.67 ±0.05	1.55 ±0.36	1.57 ±0.23	0.001	0.94	0.99

5.3.4 Physiological responses to water treatments

The photosynthetic rate of inoculated tomato plants in experiment 1 & 2 was not significantly affected by inoculum or water treatments (Figure 5.5a). Inoculated tomato plants in experiment 1 that received adequate water (AW+EW) during the deficit water period (week 21) was slightly higher than non-inoculated plants that has also same amount of water in the same period. In experiment 2, the photosynthetic rate of both inoculated and non-inoculated plants under (AW+EW), in week 10, was not different, but the photosynthetic rate of inoculated plants that received less water (DW+EW) was slightly higher than non-inoculated plants during the deficit water period. There were no significant differences due to inoculum treatment (Figure 5.5b). However, in experiment 3, results showed that photosynthetic rate of inoculated plants was significantly higher than non-inoculated (Figure 5.5c).

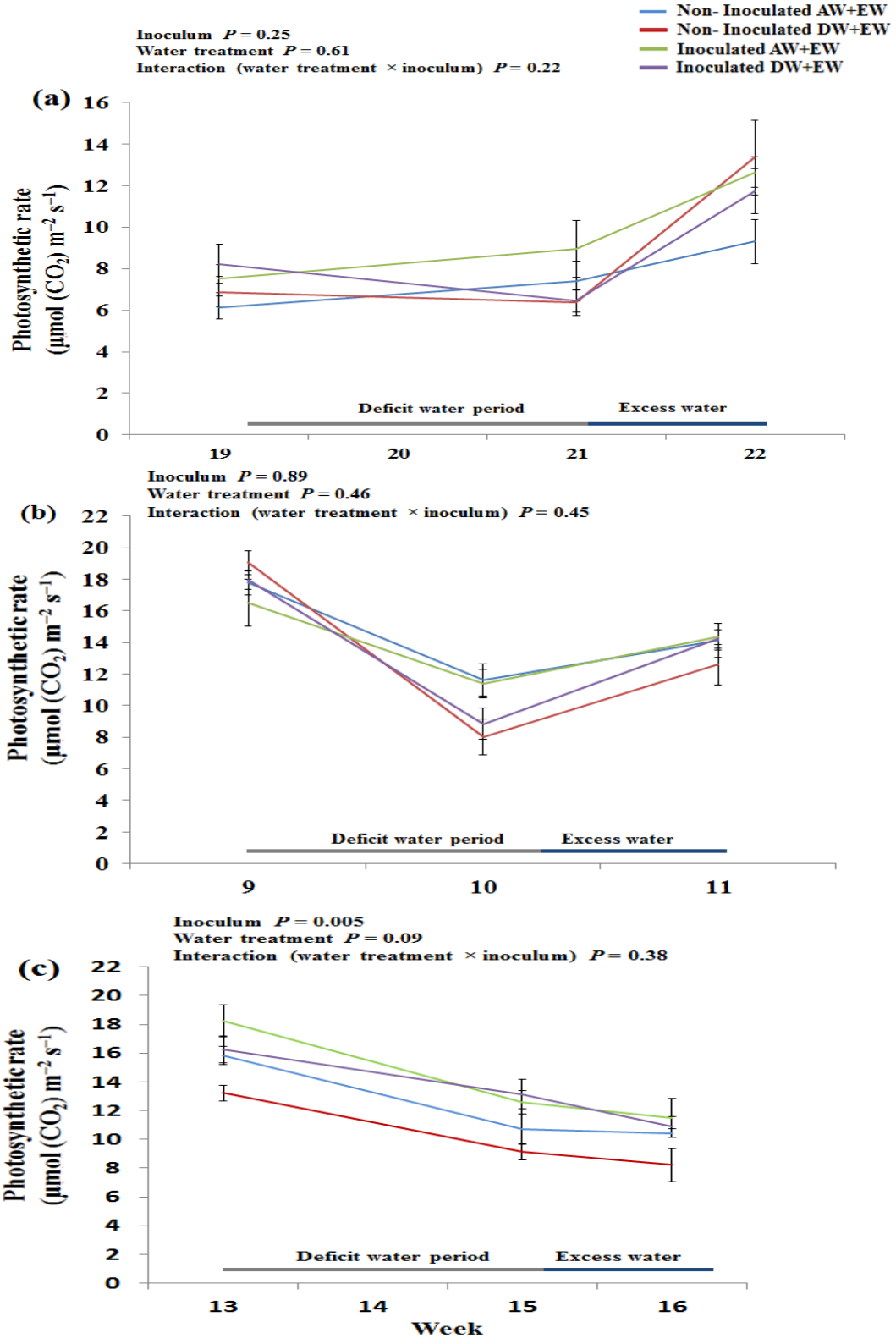

Figure 5.5: Mean value (± SE) of photosynthetic rate (A1500) (n= 20) (a) Experiment 1, (b) Experiment 2, and (c) Experiment 3. Data analysis was conducted with repeated measures ANOVA.

The functioning rate of stomatal conductance differed depending on water treatment conditions. Although on average inoculated tomato seedlings that received AW in experiment 1 had higher stomatal conductance in week 21 than non-inoculated plants under same water condition, it was not significant (Figure 5.6a). There was no significant effect of inoculum in experiment 2 (Figure 5.6 b) on stomatal conductance. However, in experiment 3 function rate of stomatal conductance of inoculated tomato leaves was significantly higher than non-inoculated under both water treatments (Figure 5.6c). Interestingly, the average stomatal conductance at all plants in experiment 3 (except the non-inoculated DW-EW plants) returned to a similar value at week 16 (Figure 5.6c).

Measurements of midday leaf water potential revealed that there were no significant effects of inoculation or water treatment in all three experiments (Figure 5.7 a, b & c).

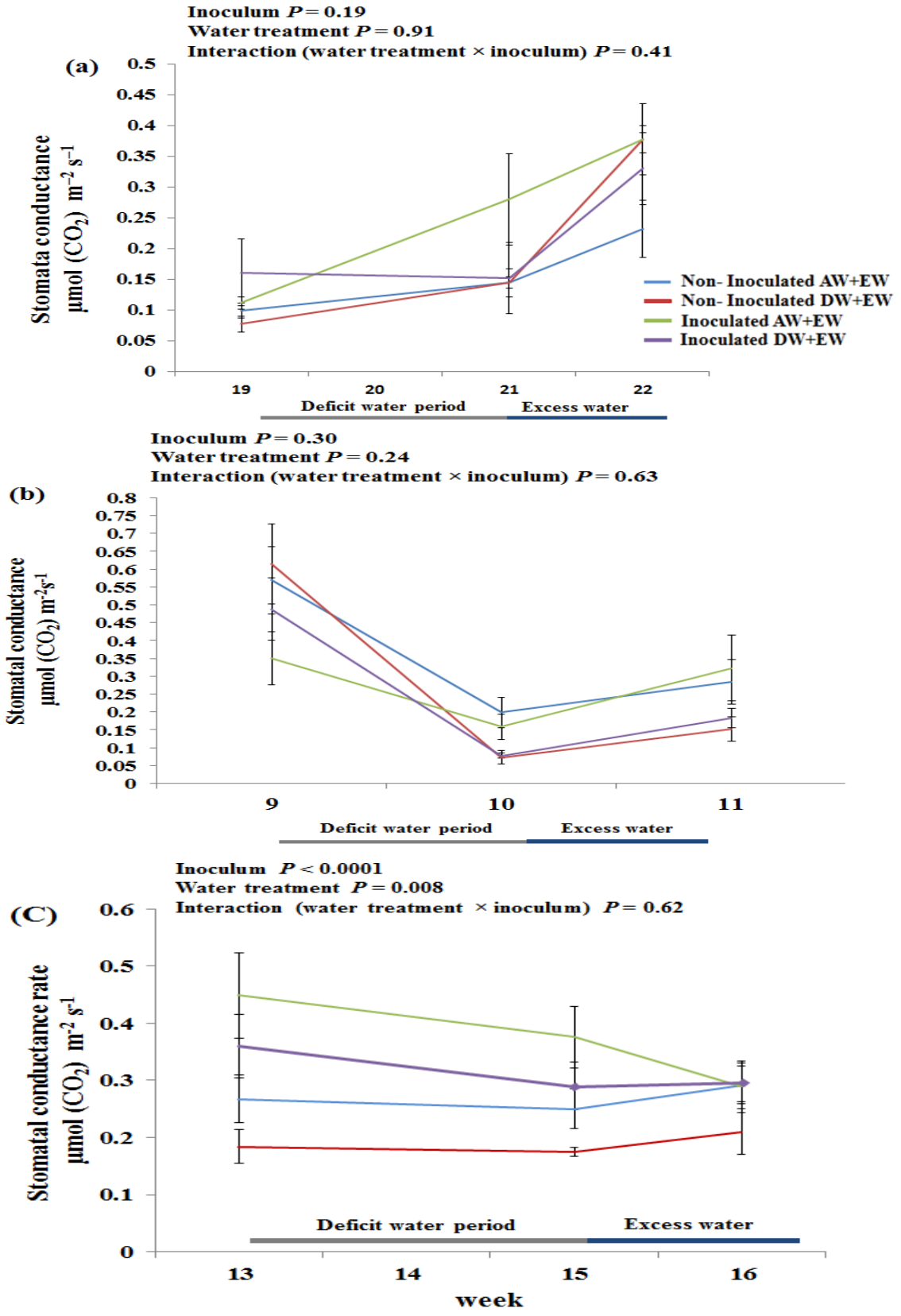

Figure 5.6: Mean value (± SE) of stomatal conductance (µmol m^{-2} s^{-1}) (n= 20) (a) Experiment 1, (b) Experiment 2, and (c) Experiment 3. Data analysis was conducted with repeated measurement ANOVA.

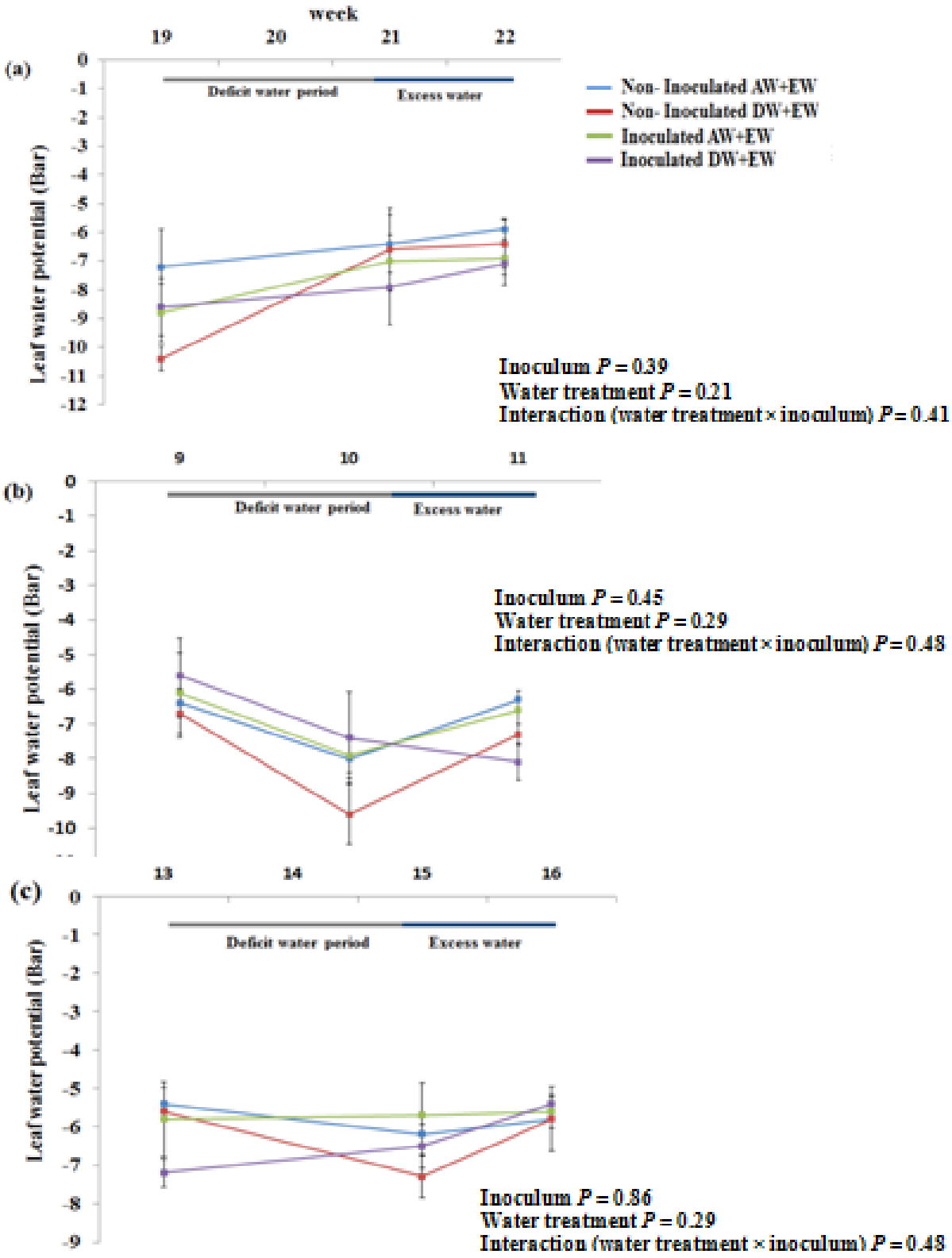

Figure 5.7: Mean value (± SE) of midday leaf water potential (Bar) (n= 20) (a) Experiment 1, (b) Experiment 2, and (c) Experiment 3. Data analysis was conducted with repeated measurement ANOVA.

5.4 Discussion

5.4.1. AMF (*R. Irregularis*) colonisation

To examine our hypotheses, it was critical that effective levels of AMF colonisation were established on inoculated plants. Good AMF colonisation was established with tomato under low phosphate in experiment 2 and 3. The percentage of AMF colonisation that occurred of the wild type tomato 76R *L. esculentum* exhibited variation from an average of 48% to 72% arbuscular presence. The structures formed by *R. irregularis* during colonisation consist of internal hyphae, vesicles and abuscules which agrees with several sources (Gao et al. 2001; Subramanian et al. 2006) who found the percentage of arbuscular presence was 82% of the *Lycopersicon esculentum* wild-type 76R roots. Low levels of root colonisation were observed in week 13 in experiment 1, which was likely due to levels of P which were inhibitory to AMF colonisation (studies have shown that high P reduces plant production of strigalactones, which stimulate AMF spore germination which agreed with other studies (Akiyama et al. 2005; Bicrniunn 1983; Foo et al. 2013; Schubert et al. 1986). Therefore the concentration of P was reduced and further spore solution was added to increase colonisation by AMF before the commencement of water treatments. This boosted colonisation of roots at the end of the experiment to levels comparable to that found for experiment 2 and 3 plants.

After harvest, a higher percentage of colonisation was measured in inoculated plant roots in experiments 1 & 3 than experiment 2. This may have been due to the soil type, as peat-perlite and vermiculite substrate was used in experiment 2, while in experiment 3 only vermiculite had been used. In addition, our study did not agree with other studies that have found peat may stimulate AMF colonisation (Bicrniunn 1983; Ma et al. 2006, 2007). Thus in experiment 2, mixed soil appeared to make tomatoes plants generally healthier; this may have reduced plant dependence on AMF by reducing production of strigolactones that play important role to establish AMF colonisation (Foo et al. 2013).

Roots of inoculated plants in experiment 3 developed the highest percentage of colonisation therefore these results are likely to be the most reliable to validly assess the hypotheses. This may be because in experiment 3 there was a low phosphate concentration for the duration of the experiment and the soil substrate encouraged reliance on AMF.

5.4.2. Effect of AMF colonisation and water stress on growth

In experiment 3, our hypothesis was supported in that *R. irregularis* inoculum did increase the height, number of leaves and stem diameter in host plants and visible and significant differences were detected compared to non-inoculated plants. This result was in accordance with the findings of Subramanian et al. (2006), who found that *Glomus clarum* enhanced the growth of inoculated tomato plants in both adequate water and drought stressed water conditions. Colonisation by AMF enhanced the plant growth parameters (shoot height, number of leaves and stem diameter) of inoculated tomato plants grown under either AW+EW or DW-EW water treatment compared with non-inoculated plants. Enhanced growth related to AMF colonisation is often attributed to improved P and other nutrient (K, N, Ca) uptake, which agreed with previous findings that indicated the core mechanism for enhancing drought tolerance was by improvement in P acquisition (Asrar et al. 2012; Augé 2004; Birhane et al. 2012; Fulton 2011; Gosling et al. 2006; Rillig et al. 1999; Schreiner et al. 2007).

AMF had positive effects on the host plant's biomass, as dry shoot and root weight of plants were significantly higher than non-inoculated plants in experiment 3, and significantly higher in shoots in experiment 2. This is consistent with other studies in which AMF colonisation increased host plant biomass (Augé 2004; Gosling et al. 2006). Moreover, the AMF plants in this study produced more root biomass than non-inoculated plants. This might partly explain why inoculated plants had a trend for higher values of leaf water potential in experiment 2 for DW plants and experiment 3 for both AW & DW plants in week 15. The ability of AMF to increase root density is consistent with earlier investigations (Al-Qarawi 2010; Berta et al. 1993). In experiment 1, the ratio of roots to shoot biomass was higher where phosphate was also elevated comparing to experiments 2 & 3. This was linked with some studies that showed amount of phosphate can affect the growth rate of the primary roots (Jiang et al. 2007; Williamson et al. 2001).

5.4.3. Effect of AMF colonisation on physiological response

AMF plants and non-AMF often display different photosynthetic characters (Borkowska 2002). Neither experiment 1 nor experiment 2 has supported our hypothesis, as no significant differences between non-inoculated and inoculated plants were found. Conversely, in experiment 3, AMF-inoculation was associated with enhanced photosynthetic rates and the stomatal conductance was also improved under different water treatments so maintaining the gas exchange (both increased CO_2 assimilation and the transpiration rate) (Augé 2004; Borkowska 2002; Subramanian et al. 2006). The findings that AMF symbiosis improved the stomatal behaviour and higher photosynthesis rate agreed with several previous studies who found AMF enhanced the stomatal behaviour to enable tolerance to water stress (Augé 2000; Gosling et al. 2006; Sheng et al. 2008).

AMF can increase water permeability in the tissues of their host plants. This helps colonized plants to maintain leaf water potentials with more water content, which can increase water under drought stress more for inoculated plants than non-inoculated plants (Augé 2004; Gosling et al. 2006; Ruiz-Lozano 2003). These results are in agreement with those reported Kaya et al. (2003) and Asrar et al. (2012) who stated that mycorrhizal colonisation might increase root length density or alter root system morphology, enabling the colonized plants to explore more soil volume and extract more water than non- inoculated plants during the drought stress.

Enhanced water conductivity of AMF colonized plants has been related to increased water uptake due to higher effective surface area (due to AMF hyphae in soil) (Augé 2001). The higher leaf water potential in inoculated tomato plants compared to non-inoculated plants grown under water stressed conditions could indicate that AMF increased the ability of roots to absorb soil moisture (Asrar et al. 2011; Asrar et al. 2012; Augé 2004; Kaya et al. 2003; Rillig et al. 1999).

5.5. Conclusion

This study found that AMF led to significant increases in growth and physiological parameters. The main objective of this study was to investigate the effectiveness of AMF on

tomato plants for better growth and improved regulation of water balance under conditions of water stress and increased tolerance in tomatoes under excess water provided by seedling inoculation with AMF than for plants without mycorrhizae.

R. irregularis inoculum did increase the height, production of leaves and stem diameter in host plants and visible and significant differences were detected compared to non-inoculated plants. AMF had positive effects on the host plant's biomass, as dry shoot and root weight of plants grown under different water treatments were significantly higher than non-inoculated plants.

AMF led to increased plant water uptake in water deficit conditions; mycorrhizal plants generally showed higher stomatal conductance and an increased rate of transpiration. Photosynthetic rates was also improved under different water treatments. Under drought stress, AMF can increase water permeability in the tissues of their host plants to maintain leaf water potentials with more water content, more for inoculated plants than non-inoculated plants.In addition, mycorrhiza affected the size of host plants, making them larger, with bigger roots that help to access a greater quantity of soil water and so scavenge water more effectively. Finally, increased tolerance in tomatoes to excess water provided by enhanced photosynthesis and keeping stomata opened by inoculated seedling.

Chapter 6 General Discussion

6.1. Comparison of tomato and sweet cherry interaction with AMF

The studies presented in this thesis have expanded our knowledge of the role of AMF in both a fast-growing herbaceous annual plant (tomato) and a slower-growing woody perennial (sweet cherry). It is pertinent to compare and contrast these two plant species in terms of their response to AMF colonisation.

6.1.1. *Rhizophagous irregularis* colonisation and effect on growth of cherry and tomato

When plants were established with low phosphate, moderate to high levels of colonisation by *R. irregularis* were obtained in sweet cherry (experiment 2) (Table 4.1a) and tomato (experiment 2 and 3) (Table 5.1b & c). This was in line with expected levels of colonisation and in agreement with previous studies (Calvet et al. 2004; Gao et al. 2001; Subramanian et al. 2006). Colonisation based on arbuscular presence was approximately double in tomato seedling roots (average 59.5% arbuscular presence for experiment 1 and 51.8% and 70.0% for experiments 2 &3 respectively) compared to cherry rootstocks (average 28.9% arbuscular presence for experiment 2 and 30.7% for experiment 3) .

It has been stated that plant dependence and responsiveness to AMF are different, and that high responsiveness cannot occur with low dependence (Janos 2007). Dependence of a plant species recognized to benefit from AMF can be determined by assessing the level of phosphorus response of plants without AMF (Janos 2007). Plant responsiveness to AMF is determined by the difference in growth between plants with and without AMF at any designated level of phosphorus availability (Janos 2007). In addition, responsiveness to AMF may be different between woody perennials and herbaceous annuals; for example the presence of AMF improved the growth of woody perennial plants under water stress more than annual plants in the fields (Jayne et al. 2014; Urcelay et al. 2003). Janos (1980) hypothesized that the mycorrhizal dependency of a plant species is related to the phase of succession, so dependence on mycorrhizae may vary with successional stage, with early-successional species being less dependent on mycorrhizas than late-successional species.

To assist the general discussion of the results presented in this thesis, plant responsiveness to mycorrhiza has been calculated based on the difference in dry biomass between plants with and without AMF by using the equation; (AMF dry weight - non-inoculated dry weight)/non-inoculated dry weight (Sawers et al. 2010). As can be seen in Table 6.1, a range of levels of responsiveness were found.

Table 6.1: The average of total above ground biomass and the level of AMF responsiveness for plants assessed in the present studies

Experiments	**Total biomass of AMF plants**	**Total biomass of non-AMF plants**	**Level of responsiveness***
Tomato experiment1	29.18	25.19	0.15
Tomato experiment2	18.92	15.85	0.19
Tomato experiment3	4.39	2.4	**0.82**
Cherry experiment2	14.55	13.07	0.11
Cherry experiment3	18.54	10.82	**0.71**

*Calculated based on Sawers et al. (2010).

All the plants in these experiments were grown in low phosphate to increase the degree of AMF dependence and the cost-benefit of relationship (Janos 2007; Tawaraya 2003). For the studies completed, sweet cherry plants were less responsive than tomato and AMF increased the growth and biomass of tomato seedlings more compared with the cherry rootstocks. Previous studies found that AMF species enhanced the growth and biomass significantly in woody plants regardless of water status such as Boswellia, Guava plantlets, *citrus tangering* and peach (*Prunus persica L. Batsch*) after establishment from seeds (Birhane et al. 2012; Estrada-Luna et al. 2000; Wu et al. 2006; Wu et al. 2011). Other studies suggested that AMF colonisation needs to occur in the early stages of growth to receive the full benefits of the association with AMF (Calvet et al. 2004; Rutto et al. 2002).

Dry shoot and root weight of plants grown under different water treatments were significantly higher than non-inoculated plants in cherry experiment 2 (Table 4.2b) and in tomato experiment 3 (Table 5.2c). This result is consistent with other studies in which increased host plant biomass was found under water stress conditions for AMF plants (Augé 2004; Gosling et al. 2006). Enhanced growth could be related to AMF colonisation as colonisation is often attributed to improved P and other nutrient (K, N, Ca) uptake; previous findings indicate the core mechanism for enhancing drought tolerance was by improvement in P acquisition (Asrar et al. 2012; Augé 2004; Birhane et al. 2012; Fulton 2011; Gosling et al. 2006; Rillig et al. 1999; Schreiner et al. 2007)**.** AMF colonisation significantly stimulated uptake of nutrients in guava plantlets and citrus tangerine seedlings, and additionally increased leaf water potential, transpiration rates, photosynthetic rates, stomatal conductance compared with that in non-AM seedlings (Estrada-Luna et al. 2000; Wu et al. 2006).

6.1.2 Effect of AMF colonisation on physiological response

Investigation of physiological aspects related to water relations and drought tolerance in AMF and non-AMF plants subjected to adequate water and drought stress has been conducted in this study. AMF symbiosis can modify plant water relations and responses to drought stress; AMF have been shown to increase transpiration rate and decrease stomatal resistance by altering the balance of plant hormones (Augé 2000). Different photosynthetic characteristics between AMF plants and non-AMF plants has been well established (Borkowska 2002). Positive effects of AMF on leaf water potential has also been seen in maize under water stress where AMF plants were able to keep stomata open longer than non-AM plants (Subramanian et al. 1995).

Tolerance to water stress, as evidenced by higher photosynthetic rate and stomatal performance, was significantly higher for tomato (herbaceous) than for cherry (woody perennial) in this study. The effect in cherry was positive, but not significant, under both water conditions. According to Augé (2001) AMF herbaceous plants (blue grama, cowpea, lettuce, rose, safflower, soybean and wheat) showed higher rates of transpiration and stomata opening relative to non-inoculated plants**.** In tomato experiments, AMF-colonisation led to improved photosynthetic rate and stomatal performance, and these also improved under water

stress, which may have occurred by increased ability of roots to absorb soil moisture, so maintaining both increased CO_2 assimilation and the transpiration rate (Augé 2004; Borkowska 2002; Subramanian et al. 2006).

AMF plants in one of the tomato experiments in this study produced more root biomass than non-inoculated plants. The ability of AMF to increase root density is consistent with earlier investigations (Al-Qarawi 2010; Berta et al. 1993). Other studies reported that mycorrhizal colonisation might increase root length density or adjust root system morphology, enabling the colonized plants to explore more soil volume and extract more water than non- inoculated plants during drought stress (Asrar et al. 2012; Kaya et al. 2003). The lack of response in the cherry seedlings may be due to the perennial nature of these plants; longer experimental periods may be required to produce greater root biomass in woody perennials.

AMF allow plants to remain more hydrated than non-inoculated plants (Augé 2001; Porcel et al. 2004), and it is possible that an increased ability for water uptake during periods of excess water may be damaging to sweet cherry production as water uptake has been demonstrated to result in fruit cracking after rainfall (Measham et al. 2010). However, as noted throughout this thesis, the presence of AMF throughout fruit development might help fruit skins maintain elasticity if water potential is mediated from extremes. A study by Balbontín et al. (2013) suggested reduced osmotic potential of fruit during rainfall may help to mitigate the development of cracking. According to many studies AMF reduced drought stress in host plants by postponed declines in leaf water potential (Augé 2001; El-Tohamy et al. 1999; Porcel et al. 2004; Song 2005). This was confirmed by this study, in cherry experiments; AMF assisted host plants to maintain a higher (less negative) leaf water potential under deficit water conditions compared with non-inoculated plants. AMF has, in other studies, increased the ability of roots to absorb soil moisture for plants growing under drought stress, which has led to increased water use efficiency compared to non-inoculated plants (Asrar et al. 2011; Asrar et al. 2012; Augé 2004; Kaya et al. 2003; Rillig et al. 1999). Enhanced water conductivity of AMF colonized plants has been related to increased water uptake due to higher effective surface area (due to AMF hyphae in soil) (Augé 2001). In addition, previous studies found that colonized plants can enhance water permeability in the tissues of host

plants which helps to maintain leaf water potentials by increased water content (Augé 2004; Gosling et al. 2006; Ruiz-Lozano 2003).

To fully answer our hypotheses that AMF plants have a reduced fruit cracking risk, trials would need to be completed in a field situation or with bigger pots, so that growth until fruiting could be obtained. Maintaining AMF-free trees in the field would be challenging and the time frame of growing trees to flowering age was beyond the scope of the current study. However, this may be a worthy future study given some promising results in the pot-studies presented.

6.2. Effect of AMF on survival in sweet cherry

Plants inoculated early with AMF are often reported to suffer less transplant shock (Section 4.3.2) , and as a result, plant survival and establishment is improved (Carpio et al. 2003; Puppi et al. 1994). According to Aka–Kacar et al. (2010), and Dolcet-Sanjuan et al. (1996), AMF inoculation can induce growth responses and plant establishment of cherry rootstocks and walnut trees. Additionally, AMF enhance Olive (*Olea europaea L.*) plants at different transplant conditions and improve transplant survival by protecting host plants against environmental stresses (Bompadre et al. 2014) such as water stress.

In our studies, inoculation with *R. irregularis* significantly increased sweet cherry establishment compared with non-inoculated plants and these results were consistent both with the hypothesis and previous findings (Gosling et al. 2006; Mukerji et al. 2000). This has positive implications for use of AMF inoculum commercially for sweet cherry to increase success of cutting survival. Our studies suggest that commercially available *R. irregularis* is a suitable choice of inoculum. Furthermore, previous studies by Calvert et al (2004) found that *G. intraradices* led to higher colonisation of *Prunus* spp. rootstocks compared to two other *Glomus* spp.

6.3 Management of AMF in commercial sweet cherry orchards

Many perennial fruit tree systems develop extensive mycorrhizal relationships, but the relationship varies among host plant species, genotype of the fungi and the abiotic and biotic context (e.g. soil nutrients, interactions with other organisms) involved in the relationship. It

has been reported that different AMF species play different roles in plant growth, because of the functional diversity of AMF; different species of mycorrhizal fungi affect plants in varying ways under different climatic and soil conditions (Carpio et al. 2003; Wu et al. 2011). A study by Morin et al. (1994) of different apple rootstock genotypes (Edabriz and Gisela 5) indicated that the rootstocks have differential preference in dependency with AMF and growing media. Although there was no effect of mycorrhizal fungus species on plant growth, *G. intraradices* plants had much higher root dry weight than control plants for 'Edabriz', while no significant effect was seen for 'Gisela 5'. As a result, the maximum benefit from AMF can be obtained from a careful selection of compatible host/fungus/substrate combinations. The performance of host plants may be greatly improved by ensuring a suitable mycorrhizal establishment at planting (Aka–Kacar et al. 2010; Azcón-Aguilar et al. 1997a).

This study provides only a preliminary characterisation of AMF abundance and diversity in organic cherry orchard soil and conventional cherry orchard soil during different seasons (autumn and spring), however some distinct trends became apparent. Better knowledge of diversity of beneficial microbes in soil, such as AMF, (and understanding which practices have a detrimental impact on AMF abundance and diversity) could lead to better orchard management and productivity. This study showed that although inoculation is possible with commercially available spores, it may be better to manage the natural inoculum. It became clear that certain AMF species could not be readily identified in the field samples; the morphological features of the spores were not distinct enough, thus use of molecular analysis to link the spore morphology analysis with genus or species would be ideal.

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